

Alterations in the intestinal microbiota of subjects diagnosed with irritable bowel syndrome

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irritable bowel syndrome**

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ACADEMIC DISSERTATION

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Abstract

Irritable bowel syndrome (IBS) is a functional bowel disorder, most likely with multiple interacting factors contributing to its aetiology. The intestinal microbiota has been proposed as one of these factors, with a putative role in the development or maintenance of IBS symptoms. The human intestinal microbiota is a rich and dynamic microbial community inhabited by 10^{14} bacteria, most of which are uncultivable. Therefore, to obtain a reliable general view of the resident bacteria and to detect specific bacterial phylotypes theoretically without restrictions, the use of molecular methods capable of detecting the uncultivable microbes is essential.

Real-time polymerase chain reaction (real-time PCR) technology was assessed for the quantification of bacteria from faecal samples, and 43 assays were designed for quantifying resident, pathogenic, probiotic or IBS-related bacteria or bacterial phylotypes. With real-time PCR, a 0.01% subpopulation could be quantified from mixed faecal deoxyribonucleic acid (DNA) samples, with a linear range of five orders of magnitude. The method proved to be sensitive and accurate also with intact bacterial cells spiked to faecal samples.

The intestinal microbiota of subjects suffering from IBS was then compared with that of healthy controls using DNA-based methods. For comparing the microbiotas on a scale covering the entire community, genomic microbial DNA extracted from faecal samples was pooled according to symptom subtype (diarrhoea-predominant, constipation-predominant, mixed-subtype) and percent guanine plus cytosine (G+C) profiled. The three most diverging %G+C fractions were analysed by cloning and partial sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene. The 16S rRNA gene sequences were used to compare differences in the subject groups in more detail. The clone libraries on the whole diverged from each other, and several differences were detected in the abundances of sequences within certain phylotypes or closely related phylotypes between various pooled samples. Sequences affiliating with the genera *Coriobacterium* and *Collinsella* within the phylum *Actinobacteria* were considerably more abundant in the pooled healthy control sample.

To analyse the quantities of these putatively IBS-associated phylotypes within individual samples, real-time PCR was applied. Several significant differences were detected, including a novel clostridial 16S rRNA gene phylotype associated with mixed-subtype IBS (IBS-M) and healthy controls and a *Ruminococcus torques* resembling phylotype associated with diarrhoea-predominant IBS (IBS-D). IBS-D patients diverged from constipation-predominant (IBS-C) and IBS-M patients and the healthy controls, in a multivariate analysis of 14 quantified phylotypes. It should be noted, however, that these results give no indication as to whether the observed alterations in the intestinal microbiotas of IBS patients are a causative agent in IBS aetiology or merely a result of the altered environment in the disturbed gut.

In conclusion, the intestinal microbiotas of IBS patients and healthy controls were found to differ from each other. These results support the hypothesis of intestinal bacteria having a role in IBS, and the specific phylotype-level differences detected warrant further studies for their potential use in IBS diagnostics, therapeutic trial follow-up and host-microbe interactions.

List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

- I Malinen E., Kassinen A., Rinttilä T. & Palva A. (2003): Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* 149:269–277.
- II Rinttilä T., Kassinen A., Malinen E., Krogius L. & Palva A. (2004): Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology* 97:1166–1177.
- III Kassinen A., Krogius-Kurikka L., Mäkituokko H., Rinttilä T., Paulin L., Corander J., Malinen E., Apajalahti J. & Palva A. (2007): The Fecal Microbiota of Irritable Bowel Syndrome Patients Differs Significantly From That of Healthy Subjects. *Gastroenterology* 133:24–33.
- IV Kassinen A., Rinttilä T., Nikkilä J., Krogius-Kurikka L., Kajander K., Malinen E., Mättö J., Mäkelä L. & Palva A.: Quantitative differences in intestinal bacterial phylotypes detected in diarrhea-predominant irritable bowel syndrome. Submitted.

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Abbreviations

ARISA	automated ribosomal intergenic spacer analysis
bp	base pair
CD	Crohn's disease
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
FISH	fluorescent <i>in situ</i> hybridization
G+C	guanine plus cytosine
GI	gastrointestinal
HITChip	Human Intestinal Tract Chip
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-C	constipation-predominant IBS
IBS-D	diarrhoea-predominant IBS
IBS-M	mixed-subtype IBS
IC	infectious colitis
MDS	multidimensional scaling
OTU	operative taxonomic unit
PAR-2	protease-activated receptor two
PCA	principal component analysis
PI-IBS	post-infectious IBS
rDNA	ribosomal deoxyribonucleic acid
RDP II	Ribosomal Database Project II
real-time PCR	real-time polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SCFA	short-chain fatty acid
SG	SYBR Green assay
SSCP	single-strand conformation polymorphism
T _m	melting temperature
TM	TaqMan assay
TGGE	temperature gradient gel electrophoresis
TRAC	transcript analysis with aid of affinity capture
TRFLP	terminal restriction fragment length polymorphism
UC	ulcerative colitis

1. Introduction

Food is digested, nutrients are absorbed and waste material is excreted through the gastrointestinal (GI) tract. Within the GI tract, a diverse and dynamic self-replicating entity essential for healthy existence resides; our intestines hold 10^{14} microbial cells, outnumbering human cells by a factor of ten (Bäckhed *et al.* 2005; Savage, 1977). The main bacterial phyla detected in the GI tract using molecular methods are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Proteobacteria* (Rajilić-Stojanović, 2007). The intestinal microbiota is relatively stable through time and individual-specific (Ley *et al.* 2006b; Matsuki *et al.* 2004; Zoetendal *et al.* 1998). Moreover, alterations in the composition of the intestinal microbiota have been associated with an impaired state of health (Dicksveld *et al.* 2008; Frank *et al.* 2007; Ley *et al.* 2006b; Malinen *et al.* 2005; Manichanh *et al.* 2006; Rajilić-Stojanović, 2007; Sokol *et al.* 2008).

Irritable bowel syndrome (IBS) is a functional bowel disorder commonly encountered world-wide (Longstreth *et al.* 2006). In Finland, the prevalence of IBS patients was 5.1% in 2001 (Hillilä & Färkkilä, 2004). The main symptoms of IBS are abdominal pain or discomfort, irregular bowel movements and constipation or diarrhoea. The syndrome is long-lasting in nature and IBS patients' well-being may be significantly affected, resulting in substantial costs to society in the form of health care visits and work absenteeism (Talley, 2008). Several possibly interacting causes of IBS have been investigated including intestinal bacteria involvement, low-grade inflammation, altered motility and sensitivity in the GI tract and psychological stress and disturbances (Drossman *et al.* 2002).

Molecular methods are often applied in studies on intestinal microbiota to target uncultivable bacteria in addition to the cultivable bacteria (Carey *et al.* 2007; Zoetendal *et al.* 2008). In this study, methods based on the guanine and cytosine percentages of microbial genomes and 16S ribosomal ribonucleic acid (rRNA) gene sequence have been applied. We aimed to design assays for analysing alterations in the intestinal microbiota of IBS patients compared with healthy controls and to apply these assays on actual samples.

1.1 Intestinal microbiota

1.1.1 Composition of the adult intestinal microbiota

From a microbial point of view, a tremendous variety of physiologically connected environments exists in the human GI tract. In the mouth, multiple distinct microbial communities consisting predominantly of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Spirochaetes*, and a candidate division TM7 are found in the saliva and plaque (Keijser *et al.* 2008).

The stomach (Figure 1), on the other hand, is believed to be comparatively resistant to microbial colonization due to acidity and digestive enzymes (Tannock, 2007), with the exception of *Helicobacter pylori* and possibly lactobacilli (Marshall & Warren, 1984; Ryan *et al.* 2008). Nevertheless, a diverse array of microbes, distinct from that of the upper parts of the GI tract, has been detected in the stomach with a culture-independent

approach, implying that the gastric microbiota has thus far been underestimated (Bik *et al.* 2006).

In the small intestine, the bacterial load and diversity rise from 10^4 to 10^8 cells per millilitre of intestinal content towards the distal ileum (Figure 1). *Bacillus*, *Lactobacillus* and *Streptococcus* species together with *Proteobacteria* predominate in the lumen of the small intestine, with the abundance of clostridia increasing in the ileum (Booijink *et al.* 2007). Reaching the colon, the transit slows down and the bacterial density rises from 10^8 in the caecum and ascending colon to an average of 10^{11} to 10^{12} cells of bacteria per gram in faeces and the proportion of obligate anaerobic bacteria increases (Figure 1). The phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* are present in the colon (Andersson *et al.* 2008; Eckburg *et al.* 2005; Gill *et al.* 2006; Hayashi *et al.* 2002a; Hayashi *et al.* 2002b; Kurokawa *et al.* 2007; Suau *et al.* 1999; Wang *et al.* 2005). In the small and large intestines, the mucosal and luminal microbiotas are distinct from each other (Booijink *et al.* 2007; Zoetendal *et al.* 2002). The main phyla detected in the human GI tract with 16S rRNA-based studies are presented in Table 1.

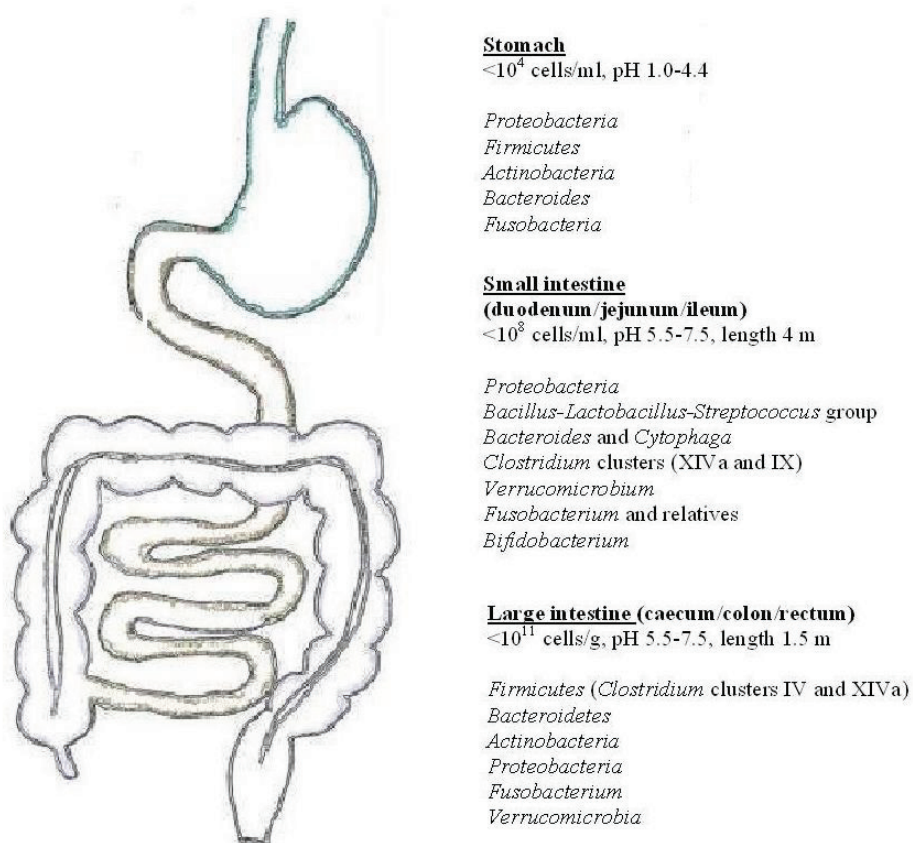


Figure 1. Characteristics of the human gastrointestinal microbiota. The average density of microbial cells, pH, lengths (Booijink *et al.* 2007) and microbial contents detected with molecular methods from the stomach (Bik *et al.* 2006), small intestine (Booijink *et al.* 2007) and large intestine (Zoetendal *et al.* 2008). Image by Erja Malinen.

Table 1. Main phyla found in the human gut in 16S rRNA gene sequencing-based studies (Rajilić-Stojanović, 2007). The abundance is given as the average detected number of bacterial cells per gram of intestinal content of the human gut. The number of phylotypes refers to the number of phylotypes detected in human gastrointestinal microbiota with culture-dependent and –independent studies during 1998–2006. Families or clusters with more than 10 detected phylotypes are presented, except for Verrucomicrobia, which is included due to its high abundance.

Phylum	Abundance	Family/Cluster	No. of phylotypes
<i>Firmicutes</i>	10^{11}	<i>Bacillaceae</i>	14
		<i>Lactobacillaceae</i>	36
		<i>Streptococcaceae</i>	32
		<i>Clostridium</i> cluster I	27
		<i>Clostridium</i> cluster IV	212
		<i>Clostridium</i> cluster IX	40
		<i>Clostridium</i> cluster XI	31
		<i>Clostridium</i> cluster XIVa	276
		Unclassified	
		<i>Clostridiales</i>	63
		<i>Clostridium</i> cluster XVI	14
<i>Bacteroidetes</i>	10^{11}	<i>Rikenellaceae</i>	15
		<i>Bacteroidaceae</i>	45
		<i>Prevotellaceae</i>	36
		<i>Porphyromonadaceae</i>	21
		Unclassified	19
<i>Actinobacteria</i>	10^{10}	<i>Bifidobacterium</i>	20
		<i>Coriobacteriaceae</i>	15
<i>Fusobacteria</i>	10^{10}	<i>Fusobacteriaceae</i>	11
<i>Verrucomicrobia</i>	10^9	<i>Verrucomicrobiaceae</i>	3
<i>Proteobacteria</i>	10^8	<i>Enterobacteraceae</i>	51
		<i>Campylobacteraceae</i>	12

1.1.2 Development of the intestinal microbiota

The first contact of a newborn with microbes occurs at birth through exposure to bacteria originating from the birth canal, the maternal faecal microbiota and the environment (Edwards & Parrett, 2002). The uniqueness of the infant intestinal microbiota is evident, as from the 136 clusters of orthologous groups enriched in infant faecal samples in a metagenomic analysis, 78 were characteristic to infants, as opposed to children and adults (Kurokawa *et al.* 2007). At the time of weaning, the GI microbiota starts to diversify, eventually resembling that of an adult (Edwards & Parrett, 2002).

The GI tract of breast-fed infants has been found to be mainly colonized by bifidobacteria, lactobacilli and streptococci, whereas formula-fed infants harbour bifidobacteria, *Bacteroidetes*, staphylococci, clostridia, *Escherichia coli* and lactobacilli (Harmsen *et al.* 2000; Penders *et al.* 2006). Contrary to the above-mentioned findings, bifidobacteria formed only a minor constituent of the GI microbiota of 14 full-term babies in the microarray study by Palmer *et al.* (2007). This could at least partially have been due to the lack of mechanical bacterial cell breakage during deoxyribonucleic acid (DNA) extraction and the high guanine plus cytosine (G+C) content of bifidobacteria causing a bias in polymerase chain reaction (Salonen *et al.* 2009).

1.1.3 Functions of the intestinal microbiota

The GI microbiota has a mutualistic relationship with its host and is involved in energy harvest from nutrients and mucus, synthesis of vitamins, metabolism of xenobiotics, renewal and differentiation of GI epithelial cells, formation of a barrier against pathogens and development and function of the immune system (Guarner, 2006; Ley *et al.* 2006a; Turnbaugh *et al.* 2007).

The principal products of microbial carbohydrate metabolism in the human GI tract are short-chain fatty acids (SCFAs), which can be absorbed by the human host. The SCFAs produced throughout the GI tract are mainly acetate, butyrate and propionate, but in the colon acetate predominates (Cummings *et al.* 1987). The colonic mucosa prefers butyrate over other SCFAs as an energy source, and butyrate has been shown to have a positive effect on health (Pryde *et al.* 2002). The most abundant intestinal butyrate-producing bacteria are *Firmicutes* from *Clostridial* clusters XIVa and IV (*Clostridium*, *Eubacterium*, *Fusobacterium*) (Pryde *et al.* 2002). Starch fermentation by starch-degrading bacteria results in comparatively high amounts of butyrate (Chassard *et al.* 2008). Starch-degrading bacteria, including *Ruminococcus bromii* (*Clostridium* cluster IV), *Eubacterium rectale* (*Clostridium* cluster XIVa) and bifidobacteria (Leitch *et al.* 2007), comprise 10.1% of culturable bacteria in faecal samples (Chassard *et al.* 2008).

Mucins are produced by the host and form a protective lubricating layer on the intestinal epithelium (Derrien, 2007) that is speculated to have a role in intestinal health (Lutgendorff *et al.* 2008). Mucins form an energy source for 5.1% of culturable intestinal bacteria (Chassard *et al.* 2008) including certain *Clostridial* cluster XIVa isolates (*Clostridium indolis* 96% and *Ruminococcus* sp.), *Bacteroides vulgatus*, several *Bifidobacteria* (*Bifidobacterium adolescentis*, *Bifidobacterium breve* and *Bifidobacterium longum*) and *Akkermansia muciniphila* (Derrien *et al.* 2004; Leitch *et al.* 2007).

1.1.4 Intestinal microbiota and health

An increasing number of studies have provided compelling evidence that the GI microbiota plays a key role in human health. The intestinal microbiota reacts to antibiotic therapies (Mellon *et al.* 2000), probiotics (Kajander *et al.* 2008; Surawicz, 2003), dietary strategies (Hayashi *et al.* 2002a) and obesity (Ley *et al.* 2006b; Turnbaugh *et al.* 2006), and it may have a role in the development of allergies (Kirjavainen *et al.* 2001; Watanabe *et al.* 2003). The normal microbiota may also contribute to the development of chronic states of recurring inflammation of the intestinal tract, such as inflammatory bowel disease (IBD), or functional intestinal disorders, such as IBS. Alterations in the composition of faecal and mucosal microbiotas (Tables 2 and 3, respectively) have been detected in both IBD, including Crohn's disease and ulcerative colitis, and IBS.

Table 2. Alterations of gut microbiota associated with IBS and IBD detected in faecal samples.

Alteration in GI microbiota	No. of subjects	Method used	Reference
Less coliforms, lactobacilli and bifidobacteria	20 IBS 20 controls	Culturing	(Balsari <i>et al.</i> 1982)
Less bifidobacteria More <i>Enterobacteriaceae</i>	25 IBS 25 controls	Culturing	(Si <i>et al.</i> 2004)
More coliforms Higher aerobe:anaerobe ratio Higher temporal instability	26 IBS 25 controls	Culturing DGGE	(Mättö <i>et al.</i> 2005)
Higher temporal instability IBS-C had less <i>Clostridium coccoides</i> - <i>Eubacterium rectale</i> group bacteria	16 IBS 16 controls	DGGE TRAC	(Maukonen <i>et al.</i> 2006)
More <i>C. coccoides</i> and <i>Bifidobacterium catenulatum</i> group bacteria IBS subtype-specific differences in <i>Lactobacillus</i> spp., <i>Veillonella</i> spp. and <i>Bifidobacterium</i> spp.	27 IBS 22 controls	Real-time PCR	(Malinen <i>et al.</i> 2005)
Distinctive clustering of phylotypes Multiple IBS subtype-specific differences in abundance of phylotypes	20 IBS 20 controls	HITChip	(Rajilić-Stojanović, 2007)
Enterobacteria observed more frequently in CD More instability	17 CD 16 controls	Dot-blot TGGE	(Seksik <i>et al.</i> 2003)
Firmicute phylotypes less diverse and less abundant	6 CD 6 controls	Metagenomic sequencing	(Manichanh <i>et al.</i> 2006)
Lower temporal stability of dominant bacteria in CD Lower diversity of lactic acid bacteria in CD	11 CD/remission 5 CD/active 18 control	DGGE	(Scanlan <i>et al.</i> 2006)
Higher proportion of bacteria detected in control subjects with 6 probes <i>Clostridium coccoides</i> group reduced in UC <i>Clostridium leptum</i> group reduced in CD <i>Bacteroides</i> group more abundant in IC	13 CD 13 UC 5 IC 13 controls	FISH	(Sokol <i>et al.</i> 2006)
Lower bacterial diversity in CD Healthy twins more similar than CD twins Alterations in abundances of <i>Bacteroides</i> species	10 CD monozygotic twin pairs 8 control monozygotic twin pairs	TRFLP %G+C profiling	(Dicksved <i>et al.</i> 2008)

CD, Crohn's disease; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization; %G+C, percent guanine plus cytosine profiling; HITChip, Human Intestinal Tract Chip; IBS, irritable bowel syndrome; IC, infectious colitis; Real-time PCR, real-time polymerase chain reaction; TGGE, temperature gradient gel electrophoresis; TRAC, transcript analysis with aid of affinity capture; TRFLP, terminal restriction fragment length polymorphism; UC, ulcerative colitis

Table 3. Alterations of gut microbiota associated with IBS and IBD detected using mucosal biopsy samples.

Alteration in GI microbiota	No. of subjects	Method used	Reference
Reduction in diversity due to loss of <i>Bacteroides</i> , <i>Eubacterium</i> and <i>Lactobacillus</i> species	57 IBD 46 controls	SSCP rDNA sequencing Real-time PCR	(Ott <i>et al.</i> 2004)
Bacteria more abundant in IBD patients <i>Bacteroides</i> dominant in IBD <i>Eubacterium rectale-Clostridium coccoides</i> group dominant in IBS	50 CD 60 non CD controls	FISH	(Swidsinski <i>et al.</i> 2005)
Distinctive clustering of DGGE fingerprints Greater variability among CD patients' microbiota <i>Clostridium</i> cluster XIVa more prevalent in CD <i>Ruminococcus torques</i> -like phylotype more prevalent in CD <i>Enterobacteriaceae</i> more prevalent in CD <i>Faecalibacterium prausnitzii</i> -like phylotype more prevalent in controls	19 CD 15 controls 1 UC 1 IC	DGGE rDNA sequencing	(Martinez-Medina <i>et al.</i> 2006)
Lower abundance of <i>Bacteroidetes</i> and higher abundance of <i>Firmicutes</i> associated with inflamed tissue of IBD patients Species richness highest in non-inflamed tissue samples of IBD patients	10 CD 15 UC 16 controls	ARISA TRFLP	(Sepehri <i>et al.</i> 2007)
<i>Faecalibacterium prausnitzii</i> associated with lower risk of postoperative relapse in CD <i>Faecalibacterium prausnitzii</i> reduced severity of colitis and balanced dysbiosis induced in mice	9 UC 9 controls	FISH Real-time PCR	(Sokol <i>et al.</i> 2008)
Clone libraries of CD, UC and non-IBD differ significantly <i>Lachnospiraceae</i> and <i>Bacteroidetes</i> less abundant in IBD	CD UC non-IBD GI patients	rDNA sequencing Real-time PCR	(Frank <i>et al.</i> 2007)
Low abundance of <i>F. prausnitzii</i> and high abundance of <i>Escherichia coli</i> associated with ileal CD	6 discordant monozyg. CD twin pairs 4 concordant monozyg. CD twin pairs	TRFLP rDNA sequencing Real-time PCR	(Willing <i>et al.</i> 2008)

ARISA, automated ribosomal intergenic spacer analysis; CD, Crohn's disease; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence in situ hybridization; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; IC, ischemic colitis; rDNA, ribosomal deoxyribonucleic acid; Real-time PCR, real-time polymerase chain reaction; SSCP, single-strand conformation polymorphism; TRFLP, terminal restriction fragment length polymorphism; UC, ulcerative colitis.

1.2 Irritable bowel syndrome (IBS)

1.2.1 Symptoms

The word “syndrome” derives from the Greek word “sundrom”, literally meaning “run together” or “meeting”. In medicine, the term refers to several features, signs, symptoms, phenomena or characteristics often occurring simultaneously. IBS is a functional disorder of the GI tract. The worldwide prevalence of IBS has been estimated to be in the range of 10-20% among adults and adolescents, depending on the diagnostic criteria applied (Longstreth *et al.* 2006). In Finland, the prevalence of IBS patients fulfilling Rome II Criteria was 5.1% of the general population in 2001 (Hillilä & Färkkilä, 2004). Abdominal pain or discomfort, irregular bowel movements and constipation or diarrhoea are common symptoms of IBS (Longstreth *et al.* 2006).

Symptoms outside the GI tract, such as fatigue, anxiety and depression, are also often encountered (Tillisch & Chang, 2005). At its worst, IBS can cause significant effects on patients’ well-being, but it is not known to predispose to any severe illnesses. Patients can be grouped into three subtypes according to bowel habits: diarrhoea-predominant (IBS-D), constipation-predominant (IBS-C) or mixed-subtype (IBS-M) (Drossman, 2006; Longstreth *et al.* 2006). However, the symptom subtype of each patient may vary over time (Longstreth *et al.* 2006).

1.2.2 Diagnostic criteria for IBS

Diagnostic symptom criteria for IBS aid in distinguishing the syndrome from organic causes and in differentiating it from other functional bowel disorders (Longstreth *et al.* 2006). Therefore, internationally agreed criteria are used in diagnosing IBS, in epidemiological surveys, in research and in therapeutic trials. The first Rome guidelines for IBS (also known as the *Rome-2 IBS Criteria*) presented in Rome in 1988 (Thompson *et al.* 1989) were based on the Manning (Manning *et al.* 1978) and Kruis (Kruis *et al.* 1984) criteria for IBS and on two epidemiological studies (Drossman *et al.* 1982; Whitehead *et al.* 1982). They were the result of the international collective effort of experts in gastroenterology chaired by W. Grant Thompson (Thompson, 2006).

Table 5. *Rome II diagnostic criteria for irritable bowel syndrome*

Rome II criteria (Thompson <i>et al.</i> 1999)
At least 6 months of recurrent symptoms of abdominal discomfort or pain <ul style="list-style-type: none"> • relieved by defecation • and/or associated with a change in stool frequency • and/or associated with a change in stool consistency
And two or more of the following: <ul style="list-style-type: none"> • altered stool frequency • altered stool form • altered stool passage • passage of mucus • bloating or feeling of abdominal distension

Thus far, Rome criteria I, II and III have been published (Longstreth *et al.* 2006; Thompson *et al.* 1992; Thompson *et al.* 1999). Rome II criteria were applied in this study (Table 4). In the more recent Rome III criteria, the temporal demands became less rigorous and the requirement for relief of abdominal symptoms with defecation was changed to improvement of symptoms. The diagnostic criteria for IBS are under constant development by specialists according to accumulating knowledge (Thompson, 2006).

1.2.3 Aetiology of IBS

Multiple theories have been proposed to explain the aetiology of IBS. These include physiological features, such as altered GI motility and visceral hypersensitivity, psychological stress and disturbances, low-grade inflammation, small intestinal bacterial overgrowth, and bacterial gastroenteritis (Drossman *et al.* 2002). Inflammation has been suggested to have a primary role in IBS aetiology; microbial infection, bacterial microbiota alteration (see following section) and abnormal response to a normal microbiota could cause low-grade inflammation, which could affect motility and visceral sensitivity (Quigley, 2006). Furthermore, environmental stress can alter GI motility and visceral sensitivity through the brain-gut axis (Quigley, 2006).

In a large cohort study (over 500 000 patients), gastroenteritis was concluded to increase the risk of developing IBS by a factor of ten (Rodriguez & Ruigomez, 1999). Post-infectious IBS (PI-IBS) has been reported after *Campylobacter*, *Shigella* and *Salmonella* infections (Ji *et al.* 2005; Mearin *et al.* 2005; Spiller *et al.* 2000), but appears to be a non-specific response (Spiller, 2007). Typically PI-IBS is characterized by loose stools, less depression and anxiety and increased enterochromaffin cells in mucosal biopsies compared with non-PI-IBS (Dunlop *et al.* 2003; Neal *et al.* 2002). Since the initial gastroenteritis triggering PI-IBS is a coincidental event, and among PI-IBS patients the symptoms are relatively homogeneous and psychological abnormalities are less common than in other IBS patients, PI-IBS presents a clearer model for studying the possible mechanisms underlying IBS (Spiller, 2007).

Elevated faecal serine protease activity has been associated with IBS-D (Roka *et al.* 2007). The faecal supernatants from IBS-D patients caused increased colonic paracellular permeability when administered to the mucosal side of a mouse colon strip and increased visceral hypersensitivity in mice (Gecse *et al.* 2008). Gecse *et al.* (2008) also showed that the effect on mucosal permeability is mediated by serine protease through protease-activated receptor two (PAR-2). Pre-incubation with serine protease inhibitors decreased the effect of the faecal supernatant from the IBS-D patients on the colonic paracellular permeability of mouse colon strips. Furthermore, the use of colonic strips derived from PAR-2-deficient mice completely removed the increase in colonic paracellular permeability. The elevated serine protease activity in IBS-D patients was suggested to be of microbial origin (Gecse *et al.* 2008).

Additionally, antibodies against certain bacterial flagellin have been detected in IBS patients, particularly in PI-IBS patients, with a higher frequency than in healthy controls (Schoepfer *et al.* 2008). Furthermore, the basal and *E. coli* lipopolysaccharide induced release of pro-inflammatory cytokines from peripheral blood mononuclear cells has been shown to be elevated in IBS-D patients compared to healthy controls (Liebregts

et al. 2007). Low-grade mucosal inflammation (Barbara *et al.* 2007; Chadwick *et al.* 2002; Dunlop *et al.* 2003; Ohman *et al.* 2005) and stable alterations in mucosal gene expression (Aerssens *et al.* 2008) of IBS patients of all symptom subtypes have also been detected. These findings, together with the IBS-associated GI microbiota alterations, imply that bacteria, host-microbe interactions and inflammation may well play a role in IBS aetiology.

1.2.3.1 Intestinal microbiota

IBS-related alterations in the GI microbiota have been investigated by conventional culture-based methods (Balsari *et al.* 1982; Mättö *et al.* 2005; Si *et al.* 2004) and molecular methods based on the 16S rRNA gene sequence (Malinen *et al.* 2005; Mättö *et al.* 2005; Maukonen *et al.* 2006; Rajilić-Stojanović, 2007; Swidsinski *et al.* 2005). Using culture-based techniques, the GI microbiota of IBS patients was characterized as having less coliforms, lactobacilli and bifidobacteria in a study with 20 IBS patients and 20 controls (Balsari *et al.* 1982). Likewise, in a later study (Si *et al.* 2004), with 25 IBS patients fulfilling the Rome II criteria and 25 controls, lower levels of bifidobacteria were detected in IBS patients, but the level of bacteria belonging to the family *Enterobacteriaceae* was higher in IBS patients. Contrary to Balsari *et al.* (1982), Mättö *et al.* (2005) detected more coliforms in IBS subjects' samples using culture-based methods.

Greater temporal instability of the intestinal microbiota of IBS patients compared with that of healthy controls has been detected with RNA-based denaturing gradient gel electrophoresis (Maukonen *et al.* 2006). The same authors also quantified the clostridial groups from the samples with a novel method, transcript analysis with aid of affinity capture (TRAC). With TRAC, IBS-C patients were found to have less *Clostridium coccoides* – *E. rectale* group bacteria than control subjects.

The sample set studied by Mättö *et al.* (2005) and Maukonen *et al.* (2006) was further studied using 20 real-time PCR assays covering approximately 300 bacterial species (27 IBS patients and 22 controls gave faecal samples at the first time-point; 21 IBS patients and 15 controls gave faecal samples at three time-points at three-month intervals) (Malinen *et al.* 2005). The first time-point was analysed with IBS subjects divided into symptom subgroups; IBS-D, IBS-C and IBS-M. Statistically significant differences were observed with real-time PCR assays targeting *Lactobacillus* spp. (less abundant in IBS-D than in IBS-C), *Veillonella* spp. (less abundant in controls than in IBS-C) and *Bifidobacterium* spp. (less abundant in IBS-D than in all other groups). The *C. coccoides* and *Bifidobacterium catenulatum* group assays detected more target bacteria in controls than in IBS patients when the results from the three different time-points were averaged and the IBS subjects analysed as a single group.

Fluorescent *in situ* hybridization (FISH) applied on mucosal samples of patients with IBD, IBS or no GI symptoms revealed that mucosal bacteria were more abundant in IBS patients than in healthy controls, although the difference was less evident than with the IBD patients (Swidsinski *et al.* 2005). The proportional amounts of the different bacterial groups targeted in the FISH analysis (*Bacteroides-Prevotella*, *B. fragilis*, *E. rectale*-*C. coccoides*, *Faecalibacterium prausnitzii* and *Enterococcus faecalis*) were similar between IBS patients and controls.

The first analysis concerning IBS-associated GI microbiota applying a microarray (The Human Intestinal Tract Chip, HITChip) was published in June 2007 in the PhD thesis of Rajilić-Stojanović (Rajilić-Stojanović, 2007). The HITChip is a 16S rRNA gene-based phylogenetic microarray specifically designed to target the human intestinal microbiota. The HITChip is unable to quantify phylotypes directly, but relative changes in hybridization signals can be detected between 0.1% and 3% subpopulations in an artificial mixture of 30 phylotypes (Rajilić-Stojanović, 2007). The HITChip study on IBS encompassed 20 IBS patients subgrouped according to symptom subtype and 20 healthy controls. With a hierarchical cluster analysis, the phylogenetic fingerprints of the faecal microbiota of IBS patients and controls grouped into two distinctive groups, with one dominated by IBS patients' samples (14 IBS patients and 4 controls) and the other by healthy controls' samples (16 controls and 6 IBS patients). The clustering did not correlate with the IBS symptom subtype. Stronger variation in the composition of the microbiota was seen among the IBS patients' profiles.

Within the phylotypes targeted by the HITChip, the IBS-C group of IBS patients had lower levels of *Bacteroides* species (*Bacteroides ovatus*, *Bacteroides uniformis*, *Bacteroides vulgatus*) and *Clostridium stercorarium*-like bacteria and higher levels of *Bacillus* spp.; the IBS-D patients were characterized by higher levels of *Aneuribacillus* spp., *Streptococcus mitis* and *Streptococcus intermedius*-like bacterial phylotypes from the order *Bacilli*. Various IBS-subgroup dependent differences were detected within *Clostridium* cluster XIVa (*C. coccoides* group). For instance, *Roseburia intestinalis* was more abundant in IBS-D and *Ruminococcus gnavus* in alternating-type IBS than in healthy controls. Several phylotypes within the *Clostridium* cluster IV (the *Clostridium leptum* -group) were more prominent in IBS-C than in IBS-D. (Rajilić-Stojanović, 2007.)

Taken together, these studies show that alterations in the GI microbiota of IBS patients compared with the microbiota of healthy controls exist and warrant further research. In particular, the application of sequencing-based approaches would yield potentially valuable IBS-associated sequence data.

1.2.3.2 Intestinal metabolites

Reduced amounts of total SCFAs due to lower levels of acetate and propionate have been measured in association with IBS-D, while an elevated concentration of n-butyrate seemed to be characteristic of IBS-D (Treem *et al.* 1996). Colonic gas production (H_2 and CH_4) has been shown to be greater in patients with IBS (Rome II criteria) compared with controls using a standardized diet, which might be associated with alterations in the activity of hydrogen-consuming bacteria (King *et al.* 1998). An exclusion diet, mainly devoid of dairy products and cereals other than rice, reduced IBS symptoms and lowered the maximum gas excretion (King *et al.* 1998). Furthermore, IBS-C has been associated with methane excretion using lactulose breath test (Pimentel *et al.* 2003).

1.2.4 Probiotics as promising remedies for IBS symptoms

In 1989, Fuller defined probiotics as “live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance” (Fuller, 1989). Probiotics have been shown to reduce IBS symptoms in several studies (for review, see Wilhelm

et al. 2008 and Spiller, 2008). Relief of bloating (Kim *et al.* 2003), flatulence (Kim *et al.* 2005), abdominal distension (Bausserman & Michail, 2005), abdominal pain and flatulence (Nobaek *et al.* 2000) and overall symptoms (Kajander *et al.* 2005; Kajander *et al.* 2008; O'Mahony *et al.* 2005) have been observed in randomized placebo-controlled intervention studies. As pointed out by Kajander in her thesis, any treatment applied as IBS therapy should be safe since the syndrome is benign (Kajander, 2008).

The intestinal microbiota has been evaluated in three probiotic interventions on IBS patients. *Lactobacillus plantarum* (DSM 9843) administered in a rose-hip drink decreased abdominal pain and flatulence (Nobaek *et al.* 2000). The abundances of *Enterobacteriaceae*, sulphate-reducing bacteria or *Enterococci* were not altered in the probiotic group, but the probiotic strain was detected in faecal and rectal mucosal samples (Nobaek *et al.* 2000). The intestinal microbiota of IBS patients receiving a multispecies probiotic consisting of *Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* Bb99 or a placebo capsule was analysed with a set of 20 real-time PCR assays targeting approximately 300 intestinal bacteria, but no effects were detected in the indigenous GI microbiota of the treatment group (Kajander *et al.* 2007). In a more recent intervention study, a similar multispecies probiotic supplement consisting of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *P. freudenreichii* ssp. *shermanii* JS and *Bifidobacterium animalis* ssp. *lactis* Bp12 was discovered to be effective in relieving IBS symptoms, especially abdominal pain and distension, and stabilizing the intestinal microbiota (Kajander *et al.* 2008).

1.3 Nucleic acid-based methods for analysing the GI microbiota

Molecular biology methods have become popular in microbial ecology studies since they do not require cultivation of microbes. The ribosomal RNA gene is especially useful due to the extensive accumulation of 16S ribosomal deoxyribonucleic acid (rDNA) sequences in public databases. The human GI microbiota can be studied with a vast array of molecular methods (Figure 2). The methods applied in this study are discussed in more detail in sections 1.3.1 to 1.3.3.

Sequencing is a prerequisite for other DNA- or RNA-based molecular methods. Cloning and sequencing of 16S rRNA gene libraries is biased as to quantity of phylotypes, but novel species can be detected and their 16S rRNA sequences utilized further with other molecular techniques (Zoetendal *et al.* 2008). Real-time PCR and FISH give quantitative results, but the target sequence has to be known beforehand. Microarray techniques are semi-quantitative and enable a vast diversity of phylotypes to be analysed in a single assay. A leap further has been taken with metagenomics, which analyses the total microbial DNA within the ecosystem of interest. Genes of various functions can be annotated from the metagenomic data in a semi-quantitative manner. Furthermore, the 16S rRNA gene sequence data in a metagenomic library can be used for phylogenetic analyses.

By the beginning of the year 2006, almost 900 rRNA gene based phylotypes originating from the human GI tract were available in public sequence databases (Rajilić-Stojanović *et al.* 2007). The richness estimates extend to 300 phylotypes within

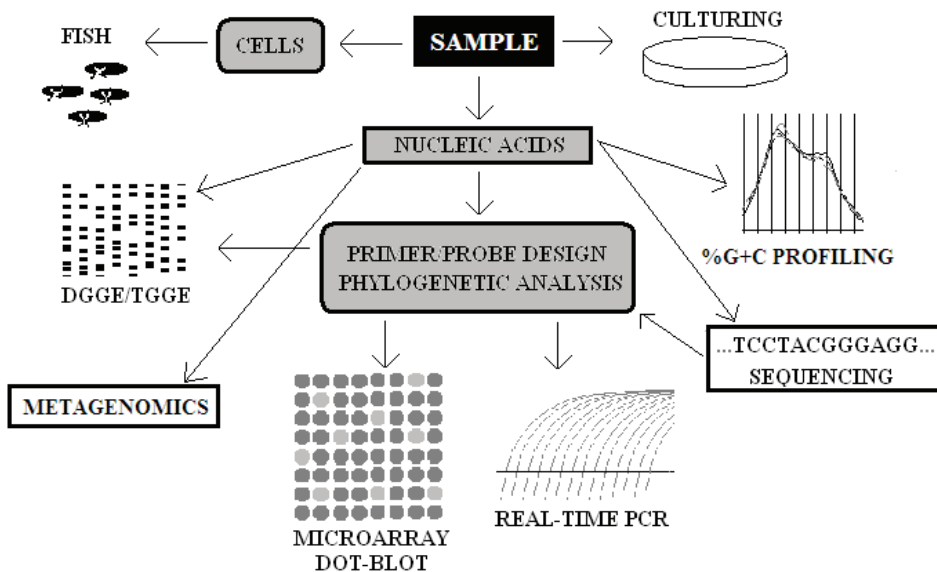


Figure 2. Nucleic acid-based methods for studying the microbiota of the GI tract. Culturing is necessary for phenotypic characterization of bacteria, whereas molecular methods can be applied to study the genotype of bacteria (sequencing, metagenomics) and the diversity (sequencing, metagenomics, DGGE/TGGE, microarray) and spatial distribution (FISH) of the microbiota. FISH, real-time PCR and dot-blot hybridization are quantitative methods, and microarray is a semi-quantitative method. Percent G+C profiling can be used to reflect the bacterial community structure. Denaturing gradient gel electrophoresis, DGGE; guanine plus cytosine, G+C; fluorescent in situ hybridization, FISH; real-time polymerase chain reaction, real-time PCR; temperature gradient gel electrophoresis, TGGE.

an individual's colon (Eckburg *et al.* 2005), with distinct disparity between individuals, indicating subject-specific variation (Eckburg *et al.* 2005; Ley *et al.* 2006b; Zoetendal *et al.* 1998). The main phyla found in 16S rRNA gene sequencing-based studies have been *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (Bonnet *et al.* 2002; Eckburg *et al.* 2005; Hayashi *et al.* 2002a; Hayashi *et al.* 2002b; Suau *et al.* 1999; Wang *et al.* 2005) (Table 1). In principle, all bacteria present in a sample can be analysed using culture-independent molecular methods. Nevertheless, the result is affected by sampling protocol, sample handling, nucleotide extraction method, selection of primers and probes and the analysis method applied.

1.3.1 Real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was developed in 1993 by Higuchi and colleagues. In the first application, the fluorescence of double-stranded DNA (dsDNA)-bound ethidium bromide was used to detect the accumulation of amplified DNA in the reaction. The original amount of target DNA in the sample can be deduced from the number of PCR cycles required to reach detectable fluorescence. Higuchi and his team were able to detect single-stranded Human Immunodeficiency Virus template DNA with a linear range

from 10^3 to 10^8 template copies (Higuchi *et al.* 1993). Real-time PCR has subsequently become a popular method in molecular biology (over 30 000 publications applying real-time PCR were available in PubMed in October 2008). An array of possible detection strategies exists that involve dsDNA intercalating dyes and sequence-specific labelled oligonucleotide probes, making multiplex real-time PCR an option (for review see Carey *et al.* 2007, Kubista *et al.* 2006 and Mackay, 2004).

In human GI research, real-time PCR has been used for detecting pathogens (Amar *et al.* 2005; Peterson *et al.* 2007; van Doornum *et al.* 2007), for quantifying specific microbial groups within the GI tract (Chen *et al.* 2007; Fite *et al.* 2004; Gueimonde *et al.* 2007; Stewart *et al.* 2006), for the microbiota as a whole (Bartosch *et al.* 2004; Hopkins *et al.* 2005; Matsuki *et al.* 2002; Penders *et al.* 2006) and in probiotic (Bartosch *et al.* 2005; Kajander *et al.* 2007) and health (Malinen *et al.* 2005; Willing *et al.* 2008) related studies.

1.3.2 Percent guanine plus cytosine (G+C) profiling

Fractionation of bacterial genomic DNA from population samples according to its genomic G+C content was developed by Holben and Harris (1995). The method was based on DNA-binding bisbenzimidazole, which preferentially binds to adenine and thymidine and changes the buoyant density of DNA proportionally to the amount of dye bound. Holben and Harris (1995) showed that the genomic DNA of *Clostridium perfringens* (27% G+C content), *E. coli* (50% G+C content) and *Micrococcus lysodeicticus* (72% G+C content) could be separated in an equilibrium density gradient caesium-chloride-bisbenzimidazole centrifugation, that the relationship between the buoyant density of DNA and the G+C content was linear and that the method was useful for bacterial community analysis of bacterial community samples.

The %G+C profiling has been shown to enrich the detectable diversity of an environmental microbial genomic DNA sample, and therefore, the method is also valuable as a preprocessing treatment (Holben *et al.* 2004; Nusslein & Tiedje, 1998). The G+C profiling method has been applied to characterization of bacterial communities in soil (Holben & Harris, 1995; Nusslein & Tiedje, 1998), and to monitoring diet-related alterations in the GI microbiota of broiler chickens (Apajalahti *et al.* 2001), the effects of inulin on the mouse caecum microbiota (Apajalahti *et al.* 2002), the effects of lactose on a colon simulator (Mäkiyuokko *et al.* 2006), and the bifidobacterial (Apajalahti *et al.* 2003) and IBD-associated (Dicksved *et al.* 2008) human GI microbiota.

1.3.3 Sequencing of 16S rRNA gene

The ca. 1500 base pair (bp) long 16S rRNA gene found in all prokaryotes can be used for estimating microbial diversity (Lozupone & Knight, 2008; Rossello-Mora & Amann, 2001; Woese, 1987). In the 16S rRNA gene primary structure, variable and highly conserved sequence regions alternate, which enable sequencing of 16S rRNA genes of unknown bacteria using universal primers annealing to the conserved parts of the gene. To date, close to one million (992 735 in November 2008) 16S rRNA entries have been submitted to public sequence databanks, constituting a large reference sequence data set. Over 15 000 of these sequences originate from human intestinal samples (Rajilić-Stojanović, 2007). One advantage of sequencing in intestinal microbiota studies is the

possibility of detecting unculturable bacteria and novel species.

The Sanger sequencing method used in this study applies dideoxy analogues of deoxynucleoside triphosphates, which terminate polymerization at a known base (Sanger *et al.* 1977). Later, the invention of pyrosequencing (Margulies *et al.* 2005) and the use of barcoded primers (Binladen *et al.* 2007) have made sequencing cost-effective and high-throughput. Andersson *et al.* (Andersson *et al.* 2008) demonstrated the applicability of barcoded 16S rDNA pyrosequencing for human intestinal microbiota. The 16S rDNA sequences can be used to estimate species richness and diversity in a microbial community (Schloss & Handelsman, 2005) and to compare different communities (Lozupone & Knight, 2008; Schloss *et al.* 2004; Schloss & Handelsman, 2006).

2. Aims of the study

- i. To test the applicability of real-time PCR for the quantification of bacteria from faecal samples (I)
- ii. To design real-time PCR assays for the quantification of an extensive set of indigenous and selected pathogenic bacterial species (I and II)
- iii. To compare the overall faecal microbiota of IBS patients and that of healthy volunteers with the whole genome and with 16S rDNA-based approaches (Figure 3; III)
- iv. To determine and quantify specific bacterial 16S rRNA gene-based phylotypes constituting differences between the faecal microbiota of IBS patients and that of healthy volunteers (Figure 3; III and IV)

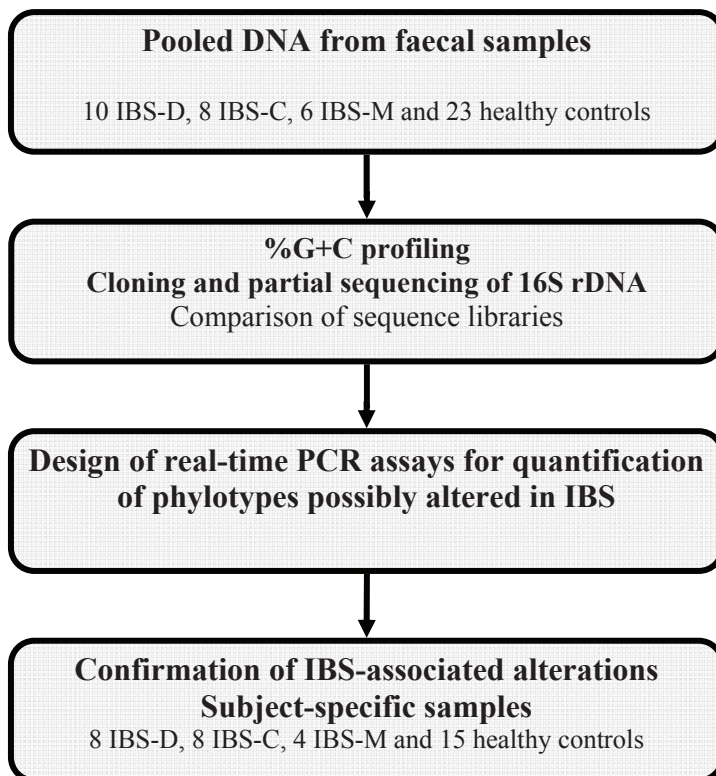


Figure 3. Study outline comparing the faecal microbiota of IBS patients with that of healthy volunteers. The %G+C profiling and analysis of partial 16S rDNA sequence libraries compare the faecal microbiotas as a whole, whereas the real-time PCR analyses on subject-specific samples are used to quantify and compare the abundances of selected phylotypes. DNA, deoxyribonucleic acid; G+C, guanine plus cytosine; IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-M, mixed-subtype IBS; real-time PCR, real-time polymerase chain reaction.

3. Materials and methods

3.1 Study subjects (II-IV)

The recruiting of all IBS patients was done by experienced physicians under the coordination of the dairy company Valio Ltd. The IBS patients analysed in this study fulfilled the Rome II criteria (Thompson *et al.* 1999), with the exception of three subjects who had slightly less than 12 weeks of abdominal pain during the preceding year (Table 5). The patients had undergone clinical investigation and endoscopy or barium enema of the GI tract within the year previous to the study.

The IBS patients had participated in a clinical probiotic intervention study (Kajander *et al.* 2007) and received daily a placebo capsule consisting of microcrystalline cellulose, magnesium stearate and gelatine as encapsulating material. Three and 24 IBS patients were analysed at one time-point (0 months) in Studies II and III, respectively. All three time-points (0, 3 and 6 months) were analysed for 20 IBS patients in Study IV.

The recruitment of a control group devoid of regular GI symptoms was coordinated by VTT Biotechnology (Espoo, Finland, Table 5). The control group was age- and gender-matched to the placebo IBS group (Mättö *et al.* 2005). The control group was also sampled at three time-points (0, 3 and 6 months). Altogether 15 control subjects completed the six-month study and were included in Study IV.

All subjects gave their written informed consent and were told that they could withdraw from the study at any time. The Human Ethics Committee at The Joint Authority for the Hospital District of Helsinki and Uusimaa (IBS patients) and the Ethics Committee of VTT (controls) approved the study protocol.

The above-mentioned samples have been analysed in multiple studies under the Finnish Funding Agency for Technology and Innovation (TEKES) project (no. 40039/03) (Malinen *et al.* 2005; Mättö *et al.* 2005; Maukonen *et al.* 2006) and by Valio Ltd. (Kajander *et al.* 2005; Kajander *et al.* 2007).

3.2 Sample handling and DNA extraction (I-V)

The faecal samples were obtained at three time-points three months apart. The subjects defecated in a plastic container made anaerobic with Anaerocult Amini (Merck, Darmstadt, Germany), whereafter the samples were immediately transported to VTT Biotechnology (Mättö *et al.* 2005). Under anaerobic conditions, the samples were homogenized by mixing with a wooden spatula and divided into subsamples. All samples were stored in -70°C prior to DNA extraction.

Extractions of DNA from bacterial cells and faecal samples were performed according to Apajalahti *et al.* (1998). Faecal bacteria were washed with repeated low-speed centrifugations after which the bacteria were pelleted with a high-speed centrifugation from the combined supernatants. The cells from the pelleted faecal bacteria (I-IV) and pure cultures of reference strains (Table 6; I-IV) were lysed with a combination of freeze-thaw cycles, lysozyme and vortexing with glass beads. The extraction method was evaluated by quantification of *E. coli* subgroup bacteria from faecal samples spiked with a dilution series of *E. coli* DSM 6897 cultures (II) and with a

Table 5. *Characteristics of IBS patients and controls.*

Characteristic	IBS patients		Controls	
Time-points (months)	0	0, 3 and 6	0	0, 3 and 6
No. of subjects	24	20	23	15
Age (years) : mean (range)	47 (21-65)	47 (24-64)	45 (24-64)	47 (25-64)
Gender: Female/Male	19/5	14/6	16/7	10/5
Predominant bowel habit				
Diarrhoea: n	10	8	-	-
Constipation: n	8	8	-	-
Alternating: n	6	4	-	-
Exclusion criteria	Antimicrobial therapy during the last two months* Pregnancy Lactation Organic gastrointestinal disease Severe systematic disease Major or complicated abdominal surgery Severe endometriosis Dementia		Regular gastrointestinal symptoms Lactose intolerance Celiac disease All exclusion criteria of the irritable bowel syndrome patients	

*Missing from Table 1 in Studies III and IV. IBS, irritable bowel syndrome.

QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturers protocol for isolating DNA from stool for pathogen detection.

3.3 Design of oligonucleotide primers and probes (I-IV)

The dot-blot hybridization probes, real-time PCR primers and TaqMan probes used in Studies I and II were designed on the basis of publicly available 16S rRNA gene sequences using ClustalW 1.83 (Thompson *et al.* 1994) for the alignment of sequences. Potential primer and probe sites were assessed either manually or using Primer3 online interface (Rozen & Skaletsky, 2000). The specificity of the probes and primers was checked with FASTA3 (Pearson & Lipman, 1988) and the Probe Match and Hierarchy Browser applications of the Ribosomal Database Project II (RDP II, RDP release 8.1) (Cole *et al.* 2003).

The 16S rRNA gene libraries constructed from the faecal samples of different IBS symptom subtype patients and healthy volunteers (III) were compared using ARB (Ludwig *et al.* 2004) and ClustalW 1.83 (Thompson *et al.* 1994) alignments. Sequences that were phylogenetically close to each other and present in diverging numbers in different clone libraries were chosen as target sequences for design of real-time PCR assays in Studies III and IV. The primers were designed using the same tools as in Studies I and II. The assays were named according to the closest cultured bacterial species with the 16S rRNA gene sequence similarity percentages below 98% indicated.

3.4 Dot-blot hybridization (I)

Dot-blot hybridizations are described in Study I. In brief, denatured DNA samples were

blotted on positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany), prehybridized with unspecific denatured DNA (Herring Sperm DNA, Sigma-Aldrich, St. Louis, MO, USA) and hybridized with oligonucleotide probes (Table 7) labelled at the 5'-end with [γ - ^{33}P] adenosine triphosphate (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After washing the membranes in stringent washing temperatures, the dots were quantified using Multi-Imager (Bio-Rad, Hercules, CA, USA).

3.5 Real-time PCR (I-IV)

For real-time PCR analyses, the iCycler iQ apparatus (Bio-Rad) associated with the ICYCLER OPTICAL SYSTEM INTERFACE software (version 2.3; Bio-Rad) was used in all four studies. For each assay, the optimal annealing temperature, MgCl_2 concentration and fluorescence measurement temperature were assessed prior to analysis of samples (Table 7). To reduce costs, the Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland) was used instead of hot-start polymerases with SYBR Green I chemistry in Studies II-IV. All samples were analysed in triplicate using 96-well optical-grade PCR plates together with standards ranging from 0.1 pg to 10 ng of genomic DNA (I and II) or 10^2 to 10^7 copies of 16S rRNA gene (III and IV). Genomic DNA of non-target GI bacteria (I and II) or the amplified 16S rRNA gene from a phylogenetically closely related clone (III and IV) was used as a negative control.

Bacterial strains were used as positive and negative controls and standards in dot-blot hybridizations and real-time PCR assays (Table 6). The media and growth conditions used are presented in the original articles.

Table 2. Bacterial strains used in Studies I-IV.

Bacterial strain ¹⁾	Study
<i>Atopobium parvulum</i> ATCC 33793	II
<i>Bacteroides fragilis</i> DSM 2151	I, II
<i>Bifidobacterium lactis</i> DSM 10140	I
<i>Bifidobacterium longum</i> DSM 20219	I-IV
<i>Campylobacter jejuni</i> Neqas 6037	II
<i>Clostridium difficile</i> ATCC9689	II
<i>Clostridium perfringens</i> ATCC 13124	II
<i>Desulfovibrio desulfuricans</i> ATCC 7757	II
<i>Enterococcus faecalis</i> DSM 20478	II
<i>Escherichia coli</i> DSM 6897	I, II
<i>Faecalibacterium prausnitzii</i> ATCC 27766	II
<i>Fusobacterium nucleatum</i> ATCC25586	II
<i>Helicobacter pylori</i> DSM 4867	II
<i>Lactobacillus acidophilus</i> ATCC 4356	I, II
<i>Lactobacillus casei</i> ATCC393	II
<i>Ruminococcus productus</i> DSM 2950	I, II
<i>Veillonella parvula</i> ATCC 10790	II

¹⁾The Department of Microbiology at the National Public Health Institute (Helsinki, Finland) and the Department of Food and Environmental Hygiene at the University of Helsinki are kindly acknowledged for providing bacterial strains.

3.5.1 Real-time PCR data analysis (I-IV)

Depending on the template used, the real-time PCR results were converted to the average estimate of bacterial genomes or 16S rRNA gene copies per 1 g of faeces (wet weight). When estimating the numbers of bacterial genomes in the faecal samples, average genome sizes for target bacteria were used and differences in the rRNA gene copy numbers were excluded. To calculate the proportional amounts of target bacteria, the quantity of eubacteria in the faecal samples were estimated with real-time PCR using universal 16S rDNA primers (Nadkarni *et al.* 2002).

For statistical comparison, the R software environment for statistical computing and graphics (R Development Core Team, 2007) was used with R-scripts for Mann-Whitney U-test (III). The Mann-Whitney U-test is a non-parametric significance test for evaluating the equality of population medians among two groups.

In Study IV, the preliminary data analysis was done using Mann-Whitney U-test (data not shown) as described above, whereas the final analyses were conducted using statistical programming language R 2.6.2 (R Development Core Team, 2008) and standard mixed-effect linear models to test the effect of time, IBS subtype and age. The model selection was based on F-tests, and the inference from the estimated models was based on standard F-tests and t-tests. Furthermore, using data from all 14 real-time PCR assays in Study IV, a principal component analysis (PCA) and hierarchical clustering (data not shown) were executed to visualize the data and to study the similarities between samples, respectively.

3.6 Percent G+C profiling of bacterial genomic DNA (III)

The %G+C profiling and fractioning of the faecal bacterial DNA samples were done according to Holben and Harris (1995) at Danisco Innovation (Kantvik, Finland). Samples were pooled based on IBS symptom subtype (10 IBS-D, 8 IBS-C, 6 IBS-M and 23 healthy controls) and centrifuged in a caesium chloride-bisbenzimidazole gradient. Thereafter, the gradient was divided into fractions at 5% G+C content intervals starting from the lower %G+C end of the profile using perfluorocarbon (fluorinert) as a piston. The amount of DNA in the profile was measured with a UV detector (A_{280}) with 1% intervals (Apajalahti *et al.* 1998). Prior to cloning and sequencing, the %G+C fractions were desalted with PD-10 columns according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB).

3.7 Cloning and sequencing (III)

From the fractions showing the most divergence between pooled samples in the %G+C profiles, 16S rRNA genes were cloned and partially sequenced. The inserts were amplified with the minimum number of cycles giving detectable bands on agarose gel electrophoresis. Two separate universal 16S rRNA gene PCR primer pairs (Hicks *et al.* 1992; Suau *et al.* 1999; Wang *et al.* 2002) were used, and the amplicons were cloned in a 1:1 molecular ratio using the Qiagen PCR Cloning Plus Kit (Qiagen).

A total of 13 824 clones were constructed and adequately stored. Of these, 4608 16S rRNA clones (384 clones from each clone library) were partially sequenced from the

5'-end of the 16S rRNA gene with a sequencing primer corresponding to the *E. coli* 16S rRNA gene positions 536–518 base pairs (bp) (Edwards *et al.* 1989). The sequencing was performed with the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3700 Capillary DNA Sequencer (GMI, Ramsey, MN, USA) at the DNA sequencing laboratory of the Institute of Biotechnology (Helsinki, Finland). After checking the quality of the sequences with a Staden program package (Staden *et al.* 2000) and excluding putative chimeras from sequences aligned with ClustalW (Chenna *et al.* 2003), the remaining 3753 sequences were deposited to the European Bioinformatics Institutes EMBL Nucleotide Sequence Database under the accession numbers AM275396-AM279148.

3.7.1 Analysing the 16S rRNA gene sequence library data (III)

The composition and structure of the clone libraries were evaluated using several methods. For the assignment of sequences into operative taxonomic units (OTUs), the sequences were aligned using ClustalW 1.83 (Thompson *et al.* 1994) (FAST DNA pairwise alignment algorithm option, Gap penalty 3, Word size 4, Number of top diagonals 1, Window size 1) and cut from the *E. coli* position 430 (totally conserved GTAAA) with BioEdit version 7.0.5.3 (Hall, 1999). Distance matrices were created with dnadist (Jukes-Cantor correction for distance) available in the Phylip 3.66 package (Felsenstein, 2005).

The OTUs were determined with DOTUR (Schloss & Handelsman, 2005) applying 98% similarity criteria. A representative sequence of each OTU was compared against the EMBL Environmental and EMBL Prokaryote DNA databases with Fasta Nucleotide Similarity Search (Pearson & Lipman, 1988). For taxonomic affiliation of sequences, the RDP II Classifier Tool (Wang *et al.* 2007) in RDP release 9 (Cole *et al.* 2007) and a phylogenetic tree with a representative sequence from each OTU and reference sequences obtained from the EMBL sequence database were used. A distance matrix constructed as previously described and the neighbour program in the Phylip 3.66 package were used for generating the tree.

The distribution of sequences originating from different samples within individual OTUs was assessed using a ClustalW 1.83 alignment of all sequences from the 12 clone libraries constructed as described above and assigning the sequences to OTUs using DOTUR. Phylogenetic differences between the clone libraries were also visualized using an ARB alignment of all sequences (Ludwig *et al.* 2004).

The β -LIBSHUFF program (Schloss *et al.* 2004), the BAPS 4.1 program for Bayesian analysis of genetic population structure (Corander *et al.* 2004; Corander & Tang, 2007) and the RDP II (RDP release 9) Library Compare Tool (Cole *et al.* 2007) were used for statistical comparison of clone libraries. The β -LIBSHUFF program analyses the differences in the libraries based on Good's formula of coverage (Good, 1953). In the Bayesian analysis, the homogeneous subgroups within the clone libraries were discovered using the unsupervised sequence classification option of BAPS 4.1 and further analysed with multidimensional scaling (MDS) (Seber, 1984). With MDS, systematic differences in the sample composition between the control and the symptom subtype can be uncovered. The RDP II Library Compare Tool assigns the sequences under comparison to phylogenetic taxa and then estimates the significance of the observed differences.

4. Results

4.1 DNA extraction

The DNA extraction method developed by Apajalahti *et al.* (1998) and the QIAamp DNA stool mini kit (Qiagen) were compared. The aforementioned method was chosen since it enables the extraction of higher molecular weight genomic DNA which is required for successful %G+C profiling (Figure 4). Furthermore, pure cultures of *E. coli* quantified using viable count were serially diluted and analysed with real-time PCR. The real-time PCR results were consistent with the viable count, giving slightly higher values with a linear range of quantification between 6.5×10^3 and 6.5×10^8 target cells quantified (II, Figure 2). This confirms that the extraction method used gives good recovery of bacterial DNA, at least in the case of *E. coli*-like Gram-negative bacteria.

4.2 Comparison of real-time PCR and dot-blot hybridization (I)

To evaluate molecular methods for quantifying bacteria from faecal samples, bacterial groups or species were quantified using dot-blot hybridization and SYBR Green I or 5'-

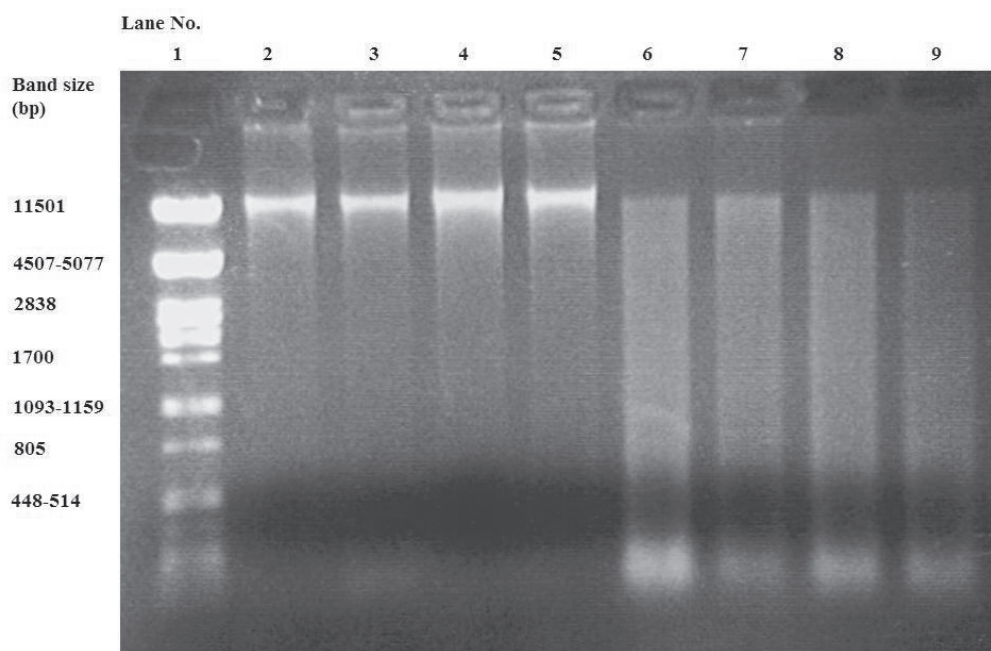


Figure 4. Faecal DNA extracted with two different extraction methods. Four parallel samples were extracted using the method described by Apajalahti *et al.*, (1998) (lanes 2-5) and the QIAamp DNA stool mini kit according to the manufacturers protocol for isolating DNA from stool for pathogen detection (QIAgen, Hilden, Germany) (lanes 6-9). The *NPstI* Marker (Fermentas, Burlington, Canada) was used (lane 1). bp, base pairs.

nuclease-based real-time PCR with 16S rRNA gene targeting primers and hybridization probes (Table 7). Dilution series of genomic DNA from pure cultures and faecal samples spiked with dilution series of test bacteria were analysed.

The sensitivity of the dot-blot hybridization method was at its best 30 ng of target genomic DNA which corresponds to approximately 10^7 target genomes (I, Table 4). Real-time PCR had a higher sensitivity and a linear range of amplification between 0.1-1 pg and 1-10 ng of target genomic DNA corresponding to approximately 200-400 and 2×10^5 - 4×10^6 target genomes (I, Figures 2 and 4). From mixed DNA samples, the dot-blot hybridization and real-time PCR (both applied chemistries) could detect a 3% and 0.01% subpopulation, respectively (I, Figures 1 and 4). The sensitivity of the methods did not change when the target DNA was mixed with faecal DNA. Furthermore, quantification of *Bifidobacterium lactis* from an artificial sample series of faeces spiked with bacterial cells of the target species was successful when 3.1×10^7 cells had been added to 1 g of faeces containing approximately 10^{11} - 10^{12} bacterial cells.

Therefore, real-time PCR was concluded to be superior in sensitivity and applicable for analysis of bacteria in faecal samples. The assay design for SYBR Green I chemistry is less troublesome since no probe sequence within the amplified area is needed.

4.3 Design of real-time PCR assays (I-IV)

Thirty-seven new real-time PCR assays for quantifying indigenous, probiotic, pathogenic or potentially IBS-associated bacteria from faecal samples were designed and optimized (Table 7). The *Lactobacillus* group (Heilig *et al.* 2002; Walter *et al.* 2001) and universal PCR primers (Nadkarni *et al.* 2002) used have been previously published. A linear range of 30-4500 to 1.9×10^6 - 6.0×10^6 target bacterial genomes (0.1-10 pg to 10 ng of bacterial genomic DNA) could be quantified using SYBR Green I-based real-time PCR, making the quantification of a 0.01% bacterial subpopulation from faecal samples possible with real-time PCR (II, Figure 1). In Studies III and IV, real-time PCR was applied successfully for comparison of IBS patients' faecal microbiota with that of healthy subjects.

Table 7. Probes and primers used in Studies I-IV.

Target species/Group (Phylum)	Control strain/Sequence	Amplicon length (bp)	T _m	Mg ²⁺ (mM)	Oligonucleotide sequence ¹⁾	Reference
Dot-blot probes						
<i>Bacteroides fragilis</i> (Bacteroidetes)	<i>B. fragilis</i> DSM 2151	-	56	-	P: GAAACATGTCAGTGAGCAATCACC	I
<i>Bifidobacterium lactis</i> (Actinobacteria)	<i>B. lactis</i> DSM 10140	-	51	-	P: GTGGAGACACGGTTTCCCTT	I
<i>Bifidobacterium longum</i> (Actinobacteria)	<i>B. longum</i> DSM 20219	-	55	-	P: GTTCCAGTTGATCGCATGGTCTT	I
<i>Escherichia coli</i> (Proteobacteria)	<i>E. coli</i> DSM 6897	-	54	-	P: GTTAATACCTTTTGCTCATTTGA	I
<i>Lactobacillus acidophilus</i> (Firmicutes)	<i>L. acidophilus</i> ATCC 4356	-	56	-	P: GATAGAGGTAGTAACCTGGCCTTTA	I
<i>Ruminococcus productus</i> (Firmicutes)	<i>R. productus</i> DSM 2950	-	54	-	P: GACATCC CTCTGACCCGTCCCGT	I
Real-time PCR primers and probes based on 16S rRNA gene sequences of bacterial species						
<i>Atopobium</i> spp. (Actinobacteria)	<i>Atopobium parvulum</i> ATCC 33793	120	61	2	F: ACCGCTTTCAGCAGGGA R: ACGCCCAATGAATCCGGAT	II
<i>Bacteroides fragilis</i> (Bacteroidetes)	<i>B. fragilis</i> DSM 2151	176	58	SG: 3 TM: 2	F: GAAAGCATTAACTATTCACCTG R: CGGTGATTGGTCACTGACA P:(HEX)-TGAAACTCAAAGGAATTGACGGGG-(DABCYL)	I
<i>Bifidobacterium lactis</i> (Actinobacteria)	<i>B. lactis</i> DSM 10140	194	58	SG: 2 TM: 1.5	F: CCCTTCCACGGGTGCC R: AAGGGAACCGTGCTCCAC P:(HEX)-AAATTGACGGGGCCCCGCACAAGC-(DABCYL)	I
<i>Bifidobacterium longum</i> (Actinobacteria)	<i>B. longum</i> DSM 20219	106	58	SG: 2.5 TM: 4	F: CAGTTGATCGCATGGTCTT R: TACCCGTGGAAGCCAC P:(FAM)-TGGGATGGGTGCGGTCTCTATCAG-(TAMRA)	I
<i>Bacteroides-Prevotella-Porphyromonas</i> (Bacteroidetes)	<i>B. fragilis</i> DSM 2151	140	68	3	F: GGTGTGGCTTAAGTGCCAT R: CGGA(C/T)GTAAAGGCCGTGC	II
<i>Bifidobacterium</i> spp.	<i>B. longum</i> DSM 20219	243	58	3	F: TGGCGTC(C/T)GGTGTGAAAG R: CCATCCACAGC(A/G)TCCAC	II
<i>Clostridium coccoideus-Eubacterium rectale</i> group (Firmicutes)	<i>R. productus</i> DSM 2950	429	55	4	F: CGGTACCTGACTAAGAAGC R: AGTTT(C/T)ATTCTTGCGAAGC	II
<i>Campylobacter</i> spp. (Proteobacteria)	<i>Campylobacter jejuni</i> Negas 6037	246	61	3	F: GGATGACACTTTTCGGAG R: AATTCATCTGCCCTCTCC	II
<i>Clostridium difficile</i> (Firmicutes)	<i>C. difficile</i> ATCC 9698	157	58	3	F: TTGAGCGATTACTCGGTAAAGA R: CCATCCTGTACTGGCTCACCT	II
<i>Clostridium perfringens</i> group (Firmicutes)	<i>C. perfringens</i> ATCC 13124	120	55	3	F: ATGCAAGTCGAGCGA(G/T)G R: TATGCGGTATTAATCT(C/T)CCTTT	II

Table 7 continuing

Target species/Group (Phylum)	Control strain/Sequence	Amplicon length (bp)	T _m	Mg ²⁺ (mM)	Oligonucleotide sequence ¹⁾	Reference
Real-time PCR primers and probes based on 16S rRNA gene sequences of bacterial species						
<i>Desulfovibrio desulfuricans</i> group (Proteobacteria)	<i>D. desulfuricans</i> ATCC 7757	191	55	4	F: GGTACCTTCAAAGGAAGCAC R: GGGATTTTCAACCCTGACTTA	II
<i>Escherichia coli</i> (Proteobacteria)	<i>E. coli</i> DSM 6897	340	60	SG: 3 TM: 6	F: GTTAATACCTTTGCTCATTTGA R: ACCAGGTATCTAATCC TGTT P:(FAM)-CGTGCCAGCAGCCGCGTA-(DABCYL)	I
<i>Enterococcus</i> spp. (Firmicutes)	<i>Enterococcus faecalis</i> DSM 20478	144	61	3	F: CCTTATTTAGTTGCCATCAATT R: ACTCGTTGTACTTCCCATTTG	II
<i>Faecalibacterium prausnitzii</i> (Firmicutes)	<i>F. prausnitzii</i> ATCC 27766	158	61	4	F: CCCTTCAGTGCCGCAGT R: GTCGCAGGATGTCAAGAC	II
<i>Fusobacterium</i> spp. (Fusobacteria)	<i>Fusobacterium nucleatum</i> ATCC 25586	273	54	5	F: (CTAT)AACGCGATAAGTAATC R: TGGTAACATACGA(AT)AGGG	II
<i>Helicobacter pylori</i> (Proteobacteria)	<i>H. pylori</i> DSM 4867	139	58	4	F: GAAGATAATGACGGTATCTAAC R: ATTTACACCTGACTGACTAT	II
<i>Helicobacter-Flexispira</i> (Proteobacteria)	<i>H. pylori</i> DSM 4867	77	61	3	F: TGGGAGAGGTAGGTGGAATTC R: GTCGCCCTTCGAATGAGTATTC	II
<i>Wolinella</i> (Proteobacteria)	<i>L. acidophilus</i> ATCC 4356	391	SG: 60 TM: 58	SG: 3 TM: 4	F: AGAGGTAGTAAGTGGCCTTTA R: GCGGAAACCTCCCAACA	I
<i>Lactobacillus acidophilus</i> (Firmicutes)	<i>L. acidophilus</i> ATCC 4356	341	58	2	P:(FAM)-CGTGCCAGCAGCCGCGTA-(DABCYL) F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	(Heilig et al, 2002; Walter et al. 2001)
<i>Ruminococcus productus</i> (Firmicutes)	<i>R. productus</i> DSM 2950	182	60	SG: 2 TM: 4	F: GGTGGCAAAAGCCATTCCGT R: GTTACGGGACGGTCAAGAG P:(HEX)-TGAAACTCAAAGGAATTGACGGGG-(DABCYL)	I
<i>Veillonella</i> spp. (Firmicutes)	<i>Veillonella parvula</i> ATCC 10790	343	62	3	F: A(C/T)CAACCTGCCCTTTCAGA R: CGTCCCATTACAGAGCTT	II
Real-time PCR primers and probes based on 16S rRNA gene phylotypes						
<i>Bacteroides intestinalis</i> -like (Bacteroidetes)	AM277809	124	63	3	F: AGCATGACCTAGCAATAGGTT R: CCTTCGTTTACTATCCGGTAT	IV
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like (Actinobacteria)	AM277149	275	68	3	F: ACTCCTCGCATGGGGTGTC R: CCGAAGGCTTGCTCCCGAT	III, IV
<i>Butyrivibrio crossotus</i> -like (Firmicutes)	AM275497	232	63	4	F: TGCATAACCCGCATATAAACAGCAGA R: CGCTGGATCAGGCTTTCCG	IV
<i>Clostridium cocleatum</i> 88% (Firmicutes)	AM276544	104	60	4	F: AATACATAAGTAACCTGGCRTC R: CGTAGCACTTTTTCATATAGATT	III, IV
<i>Clostridium thermosuccinogenes</i> 85% (Firmicutes)	AM275406	373	62	2	F: ACATGCAAGTCGAACGGAAGTC R: TGCCTCAGAGTTTCTCCTCATTG	IV

Table 7 continuing

Target species/Group (Phylum)	Control strain/Sequence	Amplicon length (bp)	T _m	Mg ²⁺ (mM)	Oligonucleotide sequence ¹⁾	Reference
Real-time PCR primers and probes based on 16S rRNA gene phylogenies						
<i>Collinsella aerofaciens</i> -like (Actinobacteria)	AM276107	260	67	4	F: CCCGACGGGAGGGGAT R: CTCTGCAGGTACAGCTTTGA	III, IV
<i>Coprobacillus cateniformis</i> 91% (Firmicutes)	AM275478	133	62	4	F: CGGACGCGATGCTTCT(A/G)GC R: AACATATCTCCATCGGGTTG	IV
<i>Coproccoccus eutactus</i> 97% (Firmicutes)	AM275825	97	63	2	F: AGCTTGCTCCGGCYGATTTA R: CGGTTTACCAGTCGTTTCCAA	III, IV
<i>Lactobacillus farciminis</i> -like (Firmicutes)	AM275648	127	63	3	F: ATGATTCAGAYCTTGGTGAG R: AAGTACGATCATGTGAAAGTA	III
<i>Lactobacillus gasseri</i> -like (Firmicutes)	AM275470	160	63	4	F: ATTTGGTGCTTGACACAGA R: CAGAACCATCTTTTAAACTCTAGA	III
<i>Ruminococcus bromii</i> -like (Firmicutes)	AM275413	156	62	4	F: CGAACGGAACTGTTTGAAGA R: CAAAACCATGCGTTCCGATAT	IV
<i>Ruminococcus torques</i> 91% (Firmicutes)	AM276558	119	62	5	F: TGCCTAACTGATCTTCTTCGGA R: CGGTATTAGCAGTCATTCTCG	III, IV
<i>Ruminococcus torques</i> 93% ²⁾ (Firmicutes)	AM275798	396	61	4	F: GACTGCTTTTGAAACTGTCA R: AGGTCCGTTAAGGA	IV
<i>Ruminococcus torques</i> 94% (Firmicutes)	AM275522	137	65	2	F: AATCTTCGGAGGAAGAGGACA R: ACATACACCATGCGGTCT	III, IV
<i>Slackia faecicantis</i> 91% (Actinobacteria)	AM276086	75	64	4	F: GAGTAACGCGTGACCGACCTT R: CCCGGAGTACCCGGTATCA	IV
<i>Spiroplasma chinense</i> 84% (Firmicutes 96%)	AM275518	101	66	4	F: ATGGCCCACTGAAGGTTG R: CCCAACGAAAGGTAGGTCA	IV
<i>Streptococcus bovis</i> -like (Firmicutes)	AM276559	150	60	5	F: TTAGCTTGCTAAAGTTGGAA R: ATCTACTAGTGAAGCAATTGCT	III
Universal real-time PCR primers						
Universal	<i>B. longum</i> DSM 20219	466	50	3	F: TCCTACGGGAGGCAGCAGT R: GGACTACAGGGTATCTAATCCTGTT	(Nadkarni et al. 2002)

¹⁾All sequences are presented from the 5' - to the 3' -end. ²⁾The sequence AY305319 (Louis et al. 2004) was used for primer design for the *Ruminococcus torques* 93% assay. F, forward primer; P, probe; R, reverse primer; SG, SYBR Green assay; TM, TaqMan assay.

4.4 Percent G+C profiling, cloning and sequencing (III)

With %G+C profiling of a bacterial community sample, the proportional amount of genomic DNA of a known %G+C content can be defined. Profiles constituted from different samples can be compared with each other in a community-level analysis. When the pooled samples of faecal microbial DNA of IBS patients (n=10 for IBS-D, n=8 for IBS-C, n=6 for IBS-M) and that of healthy controls (n=23) were %G+C profiled, the most diverging %G+C fractions were 25-30%, 40-45% and 55-60% (III, Figure 1). As the observed differences may be due to a number of bacterial species with corresponding proportional genomic G+C content, the above-mentioned diverging fractions were selected for subsequent cloning and sequencing of the 16S rRNA gene.

A total of 3753 high-quality sequences, covering approximately 450 bp from the 5'-end of the 16S rRNA, were obtained with a sequencing success rate of 81% (Table 8). The number of OTUs within each library varied from 45 to 119. When all the 3753 sequences originating from the 12 analysed clone libraries were aligned together and analysed with DOTUR, they formed 486 phylotypes (98% similarity criteria). Fiftythree phylotypes (represented by 61 %G+C fraction specific OTUs) composed of 98 sequences with less than 95% similarity to any EMBL sequence were detected (Table 8).

Table 8. Clone library characteristics.

Sample	Sequences	OTUs ¹⁾	Novel sequences ²⁾	Novel OTUs	Coverage ³⁾
Fraction %G+C 25-30					
Control	319	91	9	4	86.5
IBS-M	324	108	9	8	79.6
IBS-C	291	63	5	5	88.0
IBS-D	342	70	3	3	89.5
Fraction %G+C 40-45					
Control	346	119	9	8	80.6
IBS-M	327	100	11	6	84.7
IBS-C	323	90	4	3	86.7
IBS-D	318	78	3	3	87.9
Fraction %G+C 55-60					
Control	311	45	14	3	92.9
IBS-M	289	61	12	9	89.3
IBS-C	291	70	6	5	86.6
IBS-D	272	50	13	4	91.9

¹⁾DOTUR was used for the calculation of OTUs with 98% similarity criteria (Schloss & Handelsman, 2005). ²⁾Sequences with less than 95% sequence similarity to other public database entries were considered novel. ³⁾The coverage of clone libraries was calculated according to Good (1953).

G+C, guanine plus cytosine; OTU, operative taxonomic unit

In %G+C fractions 25-30 and 40-45, most of the sequences affiliated with *Firmicutes* (Figure 5). *Actinobacteria* outnumbered *Firmicutes* in the highest G+C content fraction analysed in all samples, except IBS-D. The genera *Coriobacterium* and *Collinsella* within the phylum *Actinobacteria* were considerably more abundant among healthy volunteers (116 sequences) than in IBS subtype patients (six, 17 and 69 sequences in clone libraries

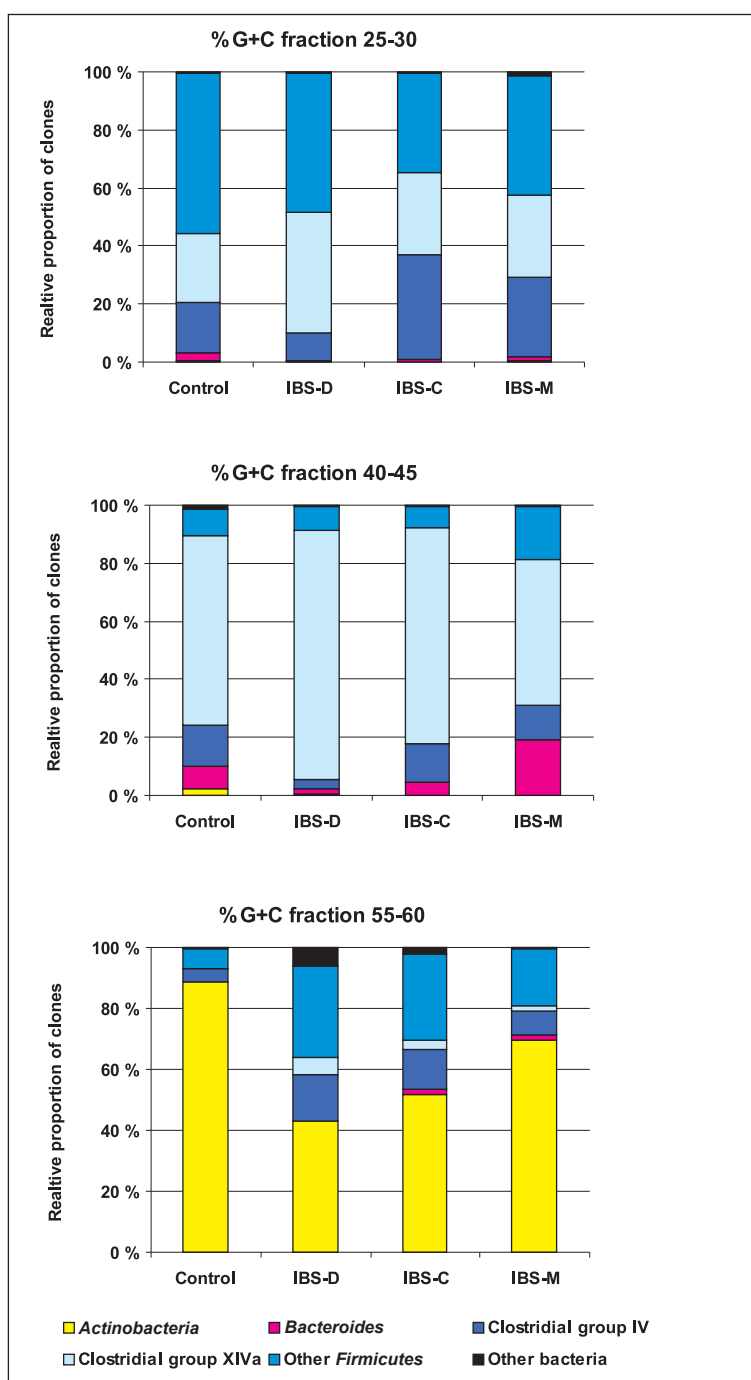


Figure 5. Affiliation of sequences derived from the 16S rRNA gene clone libraries. In fraction 25-30% G+C, sequences were affiliated with multiple clostridial groups, whereas in fraction 40-45% G+C, the *C. coccoides* group (XIVa) dominated. The highest G+C content fraction analysed (55-60%) contained mostly actinobacterial sequences in all samples, except the IBS-D sample. The relative number of sequences affiliating with Actinobacteria was greatest in the healthy controls' sample.

constructed from samples of IBS-C, IBS-D and IBS-M patients, respectively). Phylotypes with diverging numbers of sequences between sample types (IBS-D, IBS-M, IBS-C and healthy controls) were chosen as targets for real-time PCR assay design.

4.5 Comparison of sequence libraries (III)

All three statistical analyses (J-LIBSHUFF, the RDP II Library Compare Tool and the Bayesian analysis of population structure) performed on the sequence data of the 12 different libraries showed that the pooled faecal sample of healthy volunteers and the IBS subtypes differed from each other (III, Tables 5 and 6 and Figure 2). With the RDP II Library Compare Tool, significant genus-level differences were seen in the genera *Allisonella*, *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Collinsella*, *Dorea*, *Eubacterium*, *Lactobacillus*, *Lactococcus*, *Roseburia*, *Ruminococcus* and *Streptococcus* (III, Table 6).

4.6 Alterations in the faecal microbiota of IBS patients (III, IV)

To detect alterations in the GI microbiota of IBS patients at the 16S rRNA phylotype level, real-time PCR was used. The nine real-time PCR assays published in Study III were designed based on an ARB alignment of sequences data derived from IBS patients and healthy controls. The *Collinsella aerofaciens*-like, the *Clostridium cocleatum* 88% and the *Coprococcus eutactus* 97% phylotypes were significantly (p -values ≤ 0.05) more abundant in control subjects' samples ($n=22$) than in the IBS patients' samples (all symptom subtypes, $n=24$) (III, Figure 3).

In Study IV, a more thorough analysis was executed. Six real-time PCR assays published in Study III (*B. catenulatum*/*Bifidobacterium pseudocatenulatum*-like, *C. cocleatum* 88%, *C. aerofaciens*-like, *C. eutactus* 97%, *Ruminococcus torques* 91%, *R. torques* 94%) and eight novel assays (*Bacteroides intestinalis*-like, *Butyrivibrio crossotus*-like, *Clostridium thermosuccinogenes* 85%, *Coprobacillus cateniformis* 91%, *Ruminococcus bromii*-like, *R. torques* 93%, *Slackia faecicanis* 91%, *Spiroplasma chinense* 84%) were used to compare IBS patients and healthy subjects. For statistical analysis of the results, relative quantities of target 16S rRNA genes were calculated using universal real-time PCR for estimation of the total quantity of eubacterial 16S rRNA genes. All three time-points were analysed from 20 IBS patients (8 IBS-D, 8 IBS-C, and 4 IBS-M patients) and 15 healthy controls (Table 5). The *C. cateniformis* 91%, *C. cocleatum* 88%, *C. thermosuccinogenes* 85%, *R. torques* 91%, *R. torques* 93%, and *R. bromii*-like phylotypes were detected in all samples (Table 10).

In a PCA of the 14 phylotype targeting assays and three time-points (0, 3 and 6 months), IBS-D differed significantly from all other sample groups in the standard mixed-effect linear model testing of the first principal component differentiating the IBS subtypes (IV, Figure 1). The *R. torques* 94% phylotype was unique in being more predominant in IBS-D. In the assay-specific analyses, the abundances of *C. thermosuccinogenes* 85%, *R. bromii*, *R. torques* 93% and *R. torques* 94% phylotypes diverged between different IBS subtype patients and healthy subjects, showing the observed effect at all time-points analysed (Table 9). In addition, divergences were detected in time-point-dependent analyses of *B. intestinalis*-like, *C. aerofaciens*-like, *C. cocleatum* 88%, *R. torques* 91%

and *S. chinense* 84% phylotypes (IV, Supplementary Table 2). Significantly lower levels of the *C. thermosuccinogenes* 85% phylotype were associated with IBS-D patients than IBS-M patients or healthy controls. A similar trend of a higher abundance in the IBS-M and a lower abundance in IBS-D subjects' samples was observed with the *B. intestinalis*-like and *C. cocleatum* 88% phylotype targeting assays. The *B. intestinalis*-like phylotype was detected in 83% of the samples (Table 10). Furthermore, the *C. aerofaciens*-like phylotype was least abundant in the IBS-D patients' samples at the first two time-points analysed. On the other hand, the *R. torques* 94% phylotype was detected with a significantly higher abundance in IBS-D subjects' samples than in healthy controls' samples independent of the time-point analysed (Table 9).

Table 9. Average number of 16S rRNA gene copies detected with real-time PCR assays relative to the universal real-time PCR results.

Real-time PCR assay	Control	IBS-C	IBS-D	IBS-M
<i>Bacteroides intestinalis</i> -like	-4.85 ¹⁾	-4.71	-5.8	-3.46
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like	-4.1	-5.63	-5.42	-4.4
<i>Butyrivibrio crossotus</i> -like	-6.2	-6.5	-7.34	-6.04
<i>Clostridium cocleatum</i> 88%	-1.7	-2.36	-2.69	-0.72
<i>Clostridium thermosuccinogenes</i> 85%	-3.33 ^{*2)}	-3.7	-4.08 ^{*†}	-3.08 [†]
<i>Cobrobacillus cateniformis</i> 91%	-4.72	-4.41	-4.79	-4.71
<i>Collinsella aerofaciens</i> -like	-2.45	-2.9	-4.63	-1.73
<i>Coprococcus eutactus</i> 97%	-5.44	-5.91	-6.55	-4.09
<i>Ruminococcus bromii</i> -like	-3.69 [*]	-1.61 [*]	-3.4	-2.08
<i>Ruminococcus torques</i> 91%	-3.13	-2.87	-2.58	-2.83
<i>Ruminococcus torques</i> 93%	-2.41 [*]	-2.61	-2.65	-2.92 [*]
<i>Ruminococcus torques</i> 94%	-4.02 [*]	-3.39	-2.43 [*]	-3.82
<i>Slackia faecicanis</i> 91%	-5.53	-5.6	-6.22	-4.01
<i>Spiroplasma chinense</i> 84%	-5.62	-5.36	-6.51	-5.7

¹⁾Values are presented as log10 averages from three time-points (0, 3 and 6 months). ²⁾Significantly differing values (p-value ≤ 0.05) between sample types are denoted with an asterisk (*) or cross (†). IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhoea-predominant irritable bowel syndrome; IBS-M, mixed-subtype irritable bowel syndrome; real-time PCR, real-time polymerase chain reaction.

Table 10. Number of subjects with target *16S rRNA* gene copies detected with real-time PCR assays.

Real-time PCR assay	Control (n=15)	IBS-C (n=8)	IBS-D (n=8)	IBS-M (n=4)
<i>Bacteroides intestinalis</i> -like	13	6	6	4
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like	14	5	7	4
<i>Butyrivibrio crossotus</i> -like	7	0	3	2
<i>Clostridium cocleatum</i> 88%	15	8	8	4
<i>Clostridium thermosuccinogenes</i> 85%	15	8	8	4
<i>Cobrobacillus cateniformis</i> 91%	15	8	8	4
<i>Collinsella aerofaciens</i> -like	15	7	7	4
<i>Coprococcus eutactus</i> 97%	9	2	3	3
<i>Ruminococcus bromii</i> -like	15	8	8	4
<i>Ruminococcus torques</i> 91%	15	8	8	4
<i>Ruminococcus torques</i> 93%	15	8	8	4
<i>Ruminococcus torques</i> 94%	14	8	7	4
<i>Slackia faecicanis</i> 91%	8	3	4	4
<i>Spiroplasma chinense</i> 84%	9	2	5	2

IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhoea-predominant irritable bowel syndrome; IBS-M, mixed-subtype irritable bowel syndrome; real-time PCR, real-time polymerase chain reaction.

5. Discussion

The intestinal microbiota is an extremely rich and dynamic microbial community constituted mainly of not yet cultured bacteria, and therefore, molecular methods are needed for microbial community analysis (Zoetendal *et al.* 2008). Our study shows that real-time PCR is applicable for analysis of faecal samples and provides a valuable set of real-time PCR primers for quantifying human intestinal bacteria using a molecular approach. Real-time PCR has since become a popular method for quantifying intestinal bacteria (Carey *et al.* 2007), and the primers presented here have been successfully applied for analysis of faecal microbiota in IBS-related (Kajander *et al.* 2007; Malinen *et al.* 2005) and other studies (Balamurugan *et al.* 2008; Firmesse *et al.* 2007; Songjinda *et al.* 2007).

IBS, a common functional GI disorder of unknown aetiology, has a substantial impact on the patient's quality of life (Hillilä & Färkkilä, 2004; Longstreth *et al.* 2006). The diagnosis of IBS is still symptom-based and the symptoms vary between patients and over time, resulting in a heterogeneous group of IBS patients (Longstreth *et al.* 2006; Thompson *et al.* 1999). Therefore, we determined the bacterial 16S rRNA gene phylotypes differing in abundance in the faecal samples of IBS patients and healthy controls. The individuality (Ley *et al.* 2006b; Matsuki *et al.* 2004; Zoetendal *et al.* 1998) and species richness (Andersson *et al.* 2008; Eckburg *et al.* 2005) of the human intestinal microbiota add further challenges to the identification of IBS-associated 16S rRNA gene phylotypes. Molecular analyses at the level of the whole microbial community (%G+C profiling) to groups and 16S rRNA gene phylotypes of bacteria (cloning and sequencing, real-time PCR) were applied on faecal samples of IBS patients and healthy controls. Taking into account the heterogeneous nature of the sample material and the health condition in question, the results attained should be confirmed with multiple independent sample panels and subjects with separate IBS symptoms (i.e. diarrhoea, constipation) but not the whole syndrome. In addition, analysing the microbiota of mucosal samples would be worthwhile since faecal and colonic mucosal microbiotas differ (Zoetendal *et al.* 2002). However, colonoscopy is required to obtain mucosal samples, placing an extra burden on study subjects. It should also be noted that these results give no indication as to whether the observed alterations in the intestinal microbiotas of IBS patients are a causative agent in IBS aetiology or merely a result of the altered gut environment.

Moreover, with faeces as sample material, inter- and intra-individual variation is expected (Eckburg *et al.* 2005; Rajilić-Stojanović, 2007; Zoetendal *et al.* 1998). Therefore, with the aim of focusing on IBS related alterations, IBS patients were subgrouped based on symptom subtype (Thompson *et al.* 1999), and the samples were pooled accordingly prior to %G+C profiling and cloning and sequencing (III). The symptoms were assessed at the beginning of the study, making the first time-point the most reliable for symptom subgrouping. Since IBS is chronic in nature, it is unlikely that a substantial number of study subjects would have ceased to have symptoms altogether. The quantitative real-time PCR analyses (III and IV) were done using individual samples to obtain statistical power and to rule out possible biases in the pooled sample composition caused by individual samples.

Other aspects to consider are the differences in abundances and molecular features of bacterial species in faecal samples that cause biases during sample handling and nucleic acid extraction, especially for quantitative data (Salonen *et al.* 2009). In the present study, the nucleic acid extraction method described by Apajalahti *et al.*, (1998) was chosen due to %G+C profiling, and all samples were extracted using this method. The applied protocol produces a high yield of comparably high molecular weight genomic DNA necessary for %G+C density gradient centrifugation. The faecal phylotypes could possibly be more accurately quantified if the extraction method applied is verified to lyse the different bacterial species with a minimum bias (Salonen *et al.* 2009). In our study, such verification was not executed, but the quantities of predominant bacteria detected in Study II were in good agreement with previous estimations (Franks *et al.* 1998; Suau *et al.* 2001; Wilson & Blichington, 1996). The main goal in Studies III and IV was to perform comparative analyses rather than to define actual quantities.

Three subjects aged over 57 years grouped separately in a hierarchical cluster analysis of the 14 real-time PCR assays comparing IBS patients and healthy controls (data not shown). This is in accordance with earlier findings of ageing affecting the composition of the intestinal microbiota (Collado *et al.* 2007; Hayashi *et al.* 2003). Age had a significant effect in the *R. torques* 91% and a trend in the *B. catenulatum*/*B. pseudocatenulatum*-like assay results (data not shown). In the PCA done on the same data comparing IBS patients with healthy controls, the effect of age was not seen in the first two principal components. In similar studies in the future, limiting the age range of recruited subjects is advisable.

In the %G+C profiling, three fractions (%G+C 25-30, 40-45 and 55-60) were found to differ between IBS patients and healthy controls. In %G+C fraction 55-60, these differences were supported by the sequence data: the healthy control sample, which had the highest amount of genomic microbial DNA in %G+C fraction 55-60, was also most abundant with high G+C actinobacterial sequences among the 16S rDNA clone libraries. However, the distribution of genomic DNA into %G+C fractions is not absolute, and some leakage between fractions may occur. Crohn's disease patients have been investigated using %G+C profiling, but the DNA fractions were not further analysed, and no association with Crohn's disease could be seen in the %G+C profiles (Dicksved *et al.* 2008).

The clone libraries of IBS symptom subtypes and healthy controls differed from each other according to J-LIBSHUFF (Schloss *et al.* 2004), the BAPS 4.1 program for Bayesian analysis of genetic population structure (Corander *et al.* 2004; Corander & Tang, 2007) and the RDP II (RDP release 9) Library Compare Tool (Cole *et al.* 2007). In the HITChip analysis by Rajilić-Stojanović (2007), the overall microbiota of IBS patients, without subgrouping according to IBS symptom subtype, was found to differ from that of healthy volunteers. In this study, the cloning and sequencing of three %G+C fractions indicated a distinctive intestinal microbiota within each IBS symptom subtype, but the clone libraries analysed did not cover the complete microbiota, and no replicate clone libraries were analysed.

In the clone libraries, the genera *Coriobacterium* and *Collinsella* within the phylum *Actinobacteria* were considerably more abundant among healthy volunteers than among IBS subtype patients. The finding was further verified with a real-time PCR assay targeting *C. aerofaciens* in Studies III and IV. Higher levels of *Actinobacteria* have

previously been associated with healthy controls in a metagenomic study on Crohn's disease patients (Manichanh *et al.* 2006), and *C. aerofaciens* has been associated with a low risk of colon cancer (Moore & Moore, 1995). Since the samples were pooled from multiple individuals, the divergences found between clone libraries could be due to one or a few subjects distorting the composition of the pooled sample. This was proven to be the case for a *Lactobacillus farciminis*-like phylotype quantified with real-time PCR from individual samples in Study III. No replicate clone libraries could be analysed due to the high cost of sequencing at the time of the study. The clone libraries were, however, an essential tool for the design of real-time PCR assays targeting alterations in the intestinal microbiotas of IBS patients. The assays were done with individual samples.

Of the 32 tested (data not shown) real-time PCR assays designed from the IBS-related clone library sequences, this study resulted in a set of 14 assays with potentiality for differentiating the IBS-D subtype from the other IBS subtypes and the healthy controls in a multivariate analysis. This is in good accordance with other studies showing the uniqueness of IBS-D subtype in comparison to other IBS subtypes (Gecse *et al.* 2008; Roka *et al.* 2007). Therefore, it would be advisable in future studies to take the IBS symptom subtype into account.

The real-time PCR assay design in this study was based on clone library phylotypes, which makes it difficult to compare our results with previously published findings. Within the phylotypes showing divergence between sample types throughout the six month survey, *C. thermosuccinogenes* 85% represents a yet unknown firmicute within the human GI microbiota having a strong association with IBS-M and healthy controls as compared with patients suffering from IBS-D. The target sequence of the *C. thermosuccinogenes* 85% assay has previously been detected by Eckburg *et al.* (2005) and Gill *et al.* (2006).

The *R. bromii*-like phylotype was associated with IBS-C patients. *Ruminococcus bromii* related phylotypes have been shown to increase with a diet high in resistant starch (Abell *et al.* 2008). In the present study, however, it is more likely that the slowed colonic transit among IBS-C patients, rather than a dietary effect, resulted in an environment favourable for the quantified phylotype.

The *R. torques* 94% phylotype was associated with IBS-D in PCA and assay specific-analysis. The bacterium *Ruminococcus torques* is a resident member of the human GI microbiota capable of degrading mucin (Hoskins *et al.* 1985), and it has earlier been associated with the mucosa of Crohn's disease patients (Martinez-Medina *et al.* 2006). Furthermore, the specific phylotype target sequence of the *R. torques* 94% assay has previously been detected in several studies (Hayashi *et al.* 2002b; Ley *et al.* 2006b; Mai *et al.* 2006) and found to be associated with Crohn's disease (Frank *et al.* 2007). The alterations observed with *C. thermosuccinogenes* 85% and *R. torques* 94% assays were stable throughout the six-month survey. The *C. thermosuccinogenes* 85% phylotype comprised 0.05%, 0.08% and 0.01% of the faecal microbial population in the control, IBS-M and IBS-D samples, respectively, and the *R. torques* 94% phylotype comprised 0.37% and 0.01% of the IBS-D and control samples, respectively. The *R. torques* 93% phylotype was associated with the control subjects compared with IBS-M patients.

The hypothesis of the intestinal microbiota having an aetiological role in IBS is supported by the occurrence of IBS symptoms after an infectious gastroenteritis (Rodriguez & Ruigomez, 1999), the elevated amount of non-endogenous serine protease

in faecal supernatants of IBS-D patients capable of causing IBS-like symptoms in mice (Gecse *et al.* 2008), the higher levels of flagellin antibodies in IBS patients (Schoepfer *et al.* 2008) and the effectiveness of probiotic treatment in stabilizing the microbiota (Kajander *et al.* 2008). Previously, alterations at whole-community level (Rajilić-Stojanović, 2007) and evidence of divergences in specific microbial groups, species and phylotypes (Malinen *et al.* 2005; Maukonen *et al.* 2006; Rajilić-Stojanović, 2007) have been detected. The studies by Malinen *et al.* (2005) and Maukonen *et al.* (2006) were conducted using the same samples as in this study.

In the present study, the use of IBS-associated 16S rRNA gene sequence libraries for real-time PCR assay design was rewarding, as the IBS-D subtype could be differentiated by quantifying only 14 phylotypes, and phylotype-specific alterations stable over time (six-month survey) were detected.

6. Conclusions

- a) Real-time PCR proved to be a sensitive and reliable method for quantifying bacteria from faecal samples, with a linear range of quantification between 6×10^3 and 6×10^8 bacterial cells.
- b) An extensive set of real-time PCR assays targeting intestinal bacteria or phylotypes was designed. All assays presented here have been successfully applied to analyses of faecal samples. The assays based on bacterial reference strain 16S rRNA gene sequences are applicable to quantifying approximately 300 human intestinal species. They were published as the first thorough set of real-time PCR assays for quantifying the human intestinal microbiota.
- c) The clone libraries constructed from IBS symptom subtypes and healthy controls were found to diverge from each other, but no replicate clone libraries were analysed. The clone libraries provided a valuable set of sequence data for designing real-time PCR assays, specifically targeting IBS-associated alterations in the intestinal microbiota.
- d) Clone library sequence-based real-time PCR assays applied on individual samples were able to detect statistically significant differences in faecal microbiotas between IBS patients grouped according to symptom subtype and healthy controls. Whether the observed alterations are a causative agent in IBS aetiology or merely a result of the altered gut environment remains unknown. The IBS-D subtype deviated from the other IBS subtypes and healthy controls in a multivariate analysis of 14 quantified 16S rRNA gene phylotypes. A novel clostridial 16S rRNA gene phylotype, *C. thermosuccinogenes* 85%, was more strongly associated with IBS-M and healthy controls than with IBS-D, and a *R. torques* 94% phylotype was more abundant in the faecal microbiota of IBS-D patients than in that of healthy controls. Furthermore, an elevated abundance of a *R. bromii*-like phylotype and a decreased abundance of a *R. torques* 93% phylotype in compared to control subjects were associated with IBS-C and IBS-M patients, respectively. All of these phylotype-specific alterations were stable throughout the six-month survey.

7. Future aspects

This study yielded a valuable set of real-time PCR assays for evaluation of the human intestinal microbiota in general and in association with IBS or other intestinal health disturbances. The real-time PCR methodology is highly sensitive and has a comparatively wide range of nucleic acid-based quantification.

Several quantitative alterations were detected in the GI microbiotas at the 16S rRNA gene phylotype level between IBS patients subgrouped according to symptom subtype and healthy controls. The observed alterations should be tested with novel IBS patient sample panels and with subjects suffering from diarrhoea or constipation but not fulfilling IBS criteria.

Our results support the hypothesis of intestinal bacteria having a role in IBS, and the diverging phylotypes warrant further studies on their potential use in IBS diagnosis, therapeutic trial follow-up and host-microbe interactions. For more efficient diagnostic and therapeutic trial follow-up purposes, a higher through-put methodology, e.g. microarray technology or possibly multiplexing of real-time analyses, is necessary. To enable studies on host-microbe interactions of the yet uncultivated 16S rRNA phylotypes they must first be successfully isolated.

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